Molecular mechanisms of BMAA stress-response and detoxification in *Mytilus galloprovincialis*

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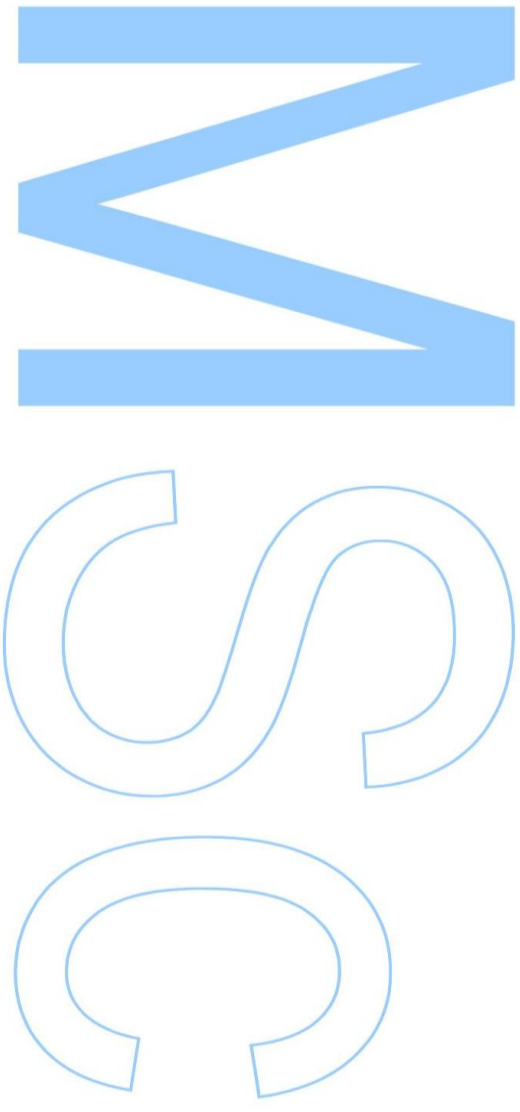

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Abstract

BMAA is a putative neurotoxin that, in marine environments, has been shown to find its way from the phytoplankton first producers (e.g. cyanobacteria) to higher trophic levels. *In vitro*, BMAA has been shown to act as an ionotropic glutamate receptor (iGluR) agonist and to induce excitotoxic effects. Despite the fact that mussels have been shown to accumulate BMAA, toxicity effects have not been yet described. This work aimed at testing the hypothesis that exposure to BMAA results in changes in the expression of iGluR, and in the activity of the enzymes Acetylcholinesterase (AChE) and Glutathione S-transferases (GSTs), in the Mediterranean mussel *Mytilus galloprovincialis*, and evaluate their potential as biomarkers of BMAA-induced toxicity.

M. galloprovincialis were exposed to 10, 100 and 1000 µg L^{-1} of BMAA standard in seawater up to 48h, and afterwards depurated until 96h. In another experiment, *M. galloprovincialis* were fed with BMAA producing cyanobacteria *Nostoc* sp, BMAA non-producing cyanobacteria *Microcoleus* sp., and the green alga *Chlorella* sp., up to 48h. Gills and digestive gland of exposed and unexposed animals were separated for enzymatic analysis and total RNA extraction. Two transcripts, termed GLU4 and GLU5, were selected from Mytibase, a catalogue of *M. galloprovincialis* expressed sequence tags (ESTs). Relative expression of the transcripts was assessed by qPCR, using the elongation factor alpha-1 (EF-1 α) as internal reference.

In mussels exposed to BMAA standard gills showed increased GSTs activity during exposure and depuration. Digestive gland also showed increased GSTs activity during exposure to BMAA. AChE activity decreased its activity during exposure to BMAA in gills, and no effects could be seen for the digestive gland. In mussels fed with cyanobacteria, both AChE and GSTs displayed an increase in activity, in digestive gland, while in gills GSTs activity decreased, and AChE activity increased. Regarding iGluR expression in mussels exposed to BMAA standard, in gills, both transcripts displayed a clear downregulation during the exposure period that was reversed after depuration, while in digestive gland results were not conclusive.

The results suggest that GSTs could be considered a potentially useful biomarker of BMAA exposure, when it is known that *M. galloprovincialis* has been exposed this amino acid, while AChE was considered a poor biomarker. The transcription of iGluR genes can potentially be used as tool to assess BMAA induced toxicity in biomonitoring studies using *M. galloprovincialis.* Nevertheless, further work is needed to better understand the regulatory mechanisms of iGLuR genes as well as

their functional role in mussels.

Resumo

BMAA é uma neurotoxina putativa que, em ambientes marinhos, foi encontrada desde entre os produtores primários fitoplanctónicos (por exemplo, cianobactérias) até aos níveis tróficos superiores. *In vitro*, foi provado que o BMAA atua como um agonista de receptores de glutamato ionotrópicos (iGluR) e induz efeitos de excitotoxicidade. Estes efeitos sobre os iGluR não foram ainda descritos em organismos marinhos, apesar do facto destes organismos terem sido reconhecidos como capazes de acumular BMAA.

Este trabalho teve como objetivo testar a hipótese de que a exposição ao BMAA resulta em alterações na expressão dos iGluR, bem como na atividade das enzimas Acetilcolinesterase (AChE) e Glutationa-S-transferases (GSTs) e avaliar o seu potencial como biomarcadores de toxicidade induzida pelo BMAA, no mexilhão mediterrânico, *Mytilus galloprovincialis*.

M. galloprovincialis foram expostos a 10, 100 e 1000 µg L^{-1} de padrão de BMAA em água do mar, durante 48h e depurados até às 96h. Noutra experiência, *M. galloprovincialis* foram alimentados com cianobactérias produtoras de BMAA, *Nostoc* sp., cianobactérias não produtoras de BMAA, *Microcoleus* sp. e com a alga verde *Chlorella* sp durante 48h. Brânquias e glândulas digestivas dos animais expostos e não expostos foram separados para análise enzimática e extracção de RNA total. Dois transcritos, denominados GLU4 e GLU5 foram selecionados a partir de um catálogo de *M. galloprovincialis* "Expressed Sequence Tag" (ESTs). A expressão relativa dos transcritos foi avaliada por qPCR, utilizando o gene do factor de alongamento alfa-1 (EF-1 α) como referência interna.

Em mexilhões expostos ao padrão BMAA, nas brânquias, observou-se uma atividade aumentada da GSTs durante a exposição e depuração. Nas glândulas digestivas também se verificou um aumento da atividade das GSTs durante a exposição ao BMAA. A atividade da AChE diminuiu durante a exposição ao BMAA nas brânquias, mas não ocorreram efeitos observáveis nas glândulas digestivas. Em mexilhões alimentados com cianobactérias, ambas AChE e GSTs apresentaram um aumento na atividade nas glândulas digestivas, enquanto nas brânquias a actividade das GSTs diminuíu, e a atividade da AChE aumentou. Em relação aos iGluR, expressos em mexilhões expostos a padrão de BMAA, nas brânquias ambos os transcritos exibiram uma regulação negativa clara durante o período de exposição, que

foi revertida após depuração, enquanto na glândula digestiva os resultados não foram conclusivos.

Os resultados sugerem que as GSTs podem ser consideradas um marcador potencialmente útil de exposição ao BMAA, quando se sabe que *M. galloprovincialis* foi exposto a este aminoácido, enquanto que a AChE foi considerado um fraco biomarcador. A expressão de genes de iGluR poderá ser utilizada como ferramenta para avaliar a toxicidade induzida por BMAA em estudos de biomonitoração de *M. galloprovincialis*. No entanto, mais estudos são necessários para entender melhor os mecanismos de regulação de genes de iGluR, bem como o seu papel funcional no mexilhão.

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List of Abbreviations

AChE - Acetylcholinesterase

AEG - N-(2-aminoethyl) glycine

aLRT - Approximate likelihood ratio test

ALS - Amyotrophic lateral sclerosis

ALS/PDC - Amyotrophic lateral sclerosis-parkinsonism-dementia complex

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA - Analysis of variance

ASP - Amnesic shellfish poisoning

BAMA - β-amino-*N*-methyl-alanine

BMAA - β-methylamino-L-alanine

BOAA - β -N-oxalylamino-L-alanine

BOGA - Biotério de Organismos Aquáticos

cDNA - Complementary deoxyribonucleic acid

CNDB - Chlorodinitrobenzene

Ct - Cycle threshold

CYN - Cylindrospermopsin

DA - Domoic acid

DAB - 2,4-Diaminobutyric Acid

DNAds - Desoxiribonucleic acid double stranded

DNAss - Desoxiribonucleic acid single stranded

DTNB - Dithiobisnitrobenzoate

DTT - Dithiothreitol

EAA - Excitatory amino acids

EDTA - Ethylenediaminetetraacetic acid

EF-1 α - Elongation factor alfa-1

ESI - Electrospray ionization

ESTs - Expressed sequence tags

EtOH - Ethanol

FU - Fluorescence

GSH - Reduced glutathione

GSTs - Glutathione s-tranferases

HAB - harmful algal blooms

HCl - Hydrochloric acid

HILIC - Hydrophilic interaction liquid chromatography

iGluR - Ionotropic glutamate receptor

IUPAC - International union of pure and applied chemistry

LC-MS/MS - Liquid chromatography - tandem mass spectrometry

LiCl – Lithium chloride

Lig_chan/Glu-bd - Glutamate binding domain

LOQ – Limit of quantification

m/z – mass/charge ratio

MAPEG - Membrane-associated proteins in eicosanoid and glutathione metabolism

MC - Microcystin

NaCl - Sodium chloride

NMDA - N-methyl-D-aspartate

NOD – Nodularin

nt - Nucleotides

ORF - Open reading frame

PBPe - Periplasmic binding protein

PBS - Phosphate buffer saline

PP - protein phosphatases

qPCR - quantitative polymerase chain reaction

RIN - Ribonucleic acid integrity number

RNAss - Ribonucleic acid single stranded

RPLC-FLD - Reversed-phase liquid chromatography with fluorescence detection

RQ - Relative quantification

SH - Shimodaira-Hasegawa

TMRs - Transmembranar

ZIC-HILIC - Zwitterionic hydrophilic interaction liquid chromatography

1. Introduction

1.1. Cyanobacteria and cyanotoxins

Cyanobacteria are photosynthetic ubiquitous micro-organisms (Sompong et al., 2005; Taton et al., 2006) as well as very important nitrogen fixing organisms that played a key role in the oxygenation of Earth's atmosphere (Paul, 2008; Bláha et al., 2009). They provide an extraordinary wide-ranging contribution to human affairs in everyday life, and are of economic importance (Bartram and Chorus, 2002). In fact, they are a source of a series of biologically active compounds with applications in areas such as medicine (Burja et al., 2001), cosmetics (Sasaki et al., 2004), and industrial production of fuel (Parmar et al., 2011)

Sometimes they produce massive growth, or blooms, mainly as a consequence of nutrient enrichment of natural waters from agricultural fields by run off, or from domestic, industrial and sewage effluents, resulting in eutrophication of the water (Codd et al. 2005, Singh et al. 2008).

Often these organisms produce various types of toxic secondary metabolites, commonly known as cyanotoxins (Sivonen and Jones, 1999). These toxins exert harmful effects on aquatic communities, and may be fatal to animals and human beings, when ingested or taken intraperitoneally (Jochimsen et al., 1998; Carmichael, 2001). Moreover, recent studies appoint global climate change as a catalyst of hazardous cyanobacterial species proliferation, persistence, dominance and activity (Bláha et al., 2009; Paerl and Huisman, 2009). Depending on their concentration in the aquatic environment, cyanotoxins can cause severe poisoning, induce chronic effects and ultimately lead to death.

There are many types of toxins, but those produced by cyanobacteria mainly fall into three categories including hepatotoxins, neurotoxins and dermatoxins (Carmichael, 1997; Sivonen and Jones, 1999; Briand et al., 2003). Predominantly, hepatotoxins affect the liver, neurotoxins affect the nervous system, and the dermatotoxins affect the skin and mucous membranes. Hepatotoxin-producing cyanobacteria are the most common (Vasconcelos et al., 1996; Chorus et al., 2000; Soares et al., 2013), although neurotoxin-producing harmful algal blooms (HAB) have been reported (Esteves et al., 1992; Batorèu et al., 2005; Bargu et al, 2012). Hepatotoxins comprise highly diverse compounds such as microcystins (MC), nodularin (NOD), and cylindrospermopsin (CYN) (Pearson, et al., 2010). On the other hand, cyanobacterial neurotoxins include alkaloid compounds such as anatoxin-a, homoanatoxin-a, anatoxin-a(s) and saxitoxins

(Aráoz et al. 2010). Exposure to cyanotoxins can occur in various ways, however contact with contaminated water or food has been considered the main route of accumulation of cyanobacterial toxins in biota, namely humans. Dermic exposure and inhalation are also possible (Ibelings and Chorus, 2007; Funari and Testai, 2008).

There have been numerous reported cases of illnesses caused by drinking or swimming in water contaminated with cyanotoxins (Teixeira et al., 1993; Falconer, 1999 Silva et al., 2014; Svirčev et al., 2014). One well-known case was in Brazil when 60 kidney patients died after being treated in a hemodyalisis clinic with water contaminated with MC (Jochimsen et al., 1998).

The literature on cyanotoxin production continues to expand, as new toxins are identified, and new tools and techniques are developed for their study. This information is useful not only in the sense of understanding the ecology and biology of cyanobacterial species, but also to provide insights on the best way of management in terms of drinking water, recreational water, and environmental health risks.

1.2. The cyanotoxin BMAA

1.2.1. History and relevance

(2S)-2-amino-3-(methylamino)propanoic acid (International Union of Pure and Applied Chemistry (IUPAC) name), also known as β -N-methylamino-L-alanine (BMAA), is a methylated non-proteinogenic amino acid first isolated by Vega and Bell (1967) from cicads *Cycas* sp., in the island of Guam (Pacific Ocean).

After the Second World War, an extremely high rate of incidence of amyotrophic lateral sclerosis/parkinsonism–dementia complex (ALS/PDC), a fatal neuromuscular condition characterized by symptoms such as paralysis, shaking, and dementia (Plato, 2003) was recorded, amongst the Chamorro people of Guam (Pacific Ocean), and thus began the search for environmental agents that could potentially be involved in the etiology of this disease (Cox et al., 2003). A small percentage of the cases ALS/PDC are considered hereditary, half of which are a result of a superoxide dismutase mutation (SOD-1) (Rosen et al., 1993). However, the great majority of ALS cases occur sporadically, with no apparent connection to family history and environmental factors have been considered as potential etiological agents. So far it has not been identified any cause for sporadic ALS (Banack et al., 2010).

Livestock neurological symptoms were associated with foraging of Cycads. Considering these findings and the common consumption of cycad seeds as flour by

the indigenous Chamorro people, a link with ALS/PDC was suggested (Whiting et al., 1966). After isolation in cycad tissues BMAA was put forward as being a possible cause of ALS/PDC in the Chamorro people of Guam (Vega and Bell, 1967).

Parallel research showed that injection of BMAA into chicks and rats caused convulsions, supporting the hypothesis of neurotoxic behavior of this compound (Vega and Bell, 1967; Vega and Bell, 1968). However, dietary exposure to the toxin was not sufficient to induce long term effects in macaques (Spencer et al., 1987) whereas ALS/PDC took years or even decades after exposure to become symptomatic. Moreover, cycad seeds processing was suggested to remove over 80% of BMAA, which implied extremely high doses of the toxin would have to be consumed to induce neurotoxic effects. Consequently, BMAA was questioned as causative agent of ALS (Duncan et al., 1990).

Only later, when the amino acid was found to be biomagnified in animals, which forage on cycad seeds, and are subsequently consumed by the Chamorro people did the hypothesis of BMAA neurotoxicity re-emerge (Charlton et al., 1992). BMAA biomagnification was reported when an increased concentration of the toxicant along the Guam food chain was observed. Samples of Mariana flying fox (*Pteropus mariannus mariannus*) skin presented BMAA levels three times more elevated than the cycad seed coat eaten by them (Murch et al. 2004). Flying foxes were part of the traditional diet of the Chamorro people, which suggested that BMAA was consumed by humans at much higher concentrations than previously thought. This toxin was then identified in the brains of deceased ALS/PDC patients from Guam (Cox et al., 2003).

Later, BMAA was also identified in the brain of patients in Canada and USA, meaning that the biomagnification hypothesis of BMAA may be more widespread than initially thought and not exclusive to Guam (Cox et al., 2003; Banack et al., 2006). However, these results were subjected to some controversy as they could not be replicated by other research group (Montine et al., 2005; Snyder et al., 2009).

1.2.2. Presence in cyanobacteria

BMAA presence in symbiotic *Nostoc* sp. that colonized the coralloid roots of the *Cycas* sp. was the first report in cyanobacteria (Cox et al., 2003).

This discovery prompted the screening of cyanobacteria from all over the world for BMAA. Since then, BMAA has been found in several other strains of cyanobacteria, as symbiont or as free-living species, in terrestrial, marine, brackish or freshwater environments (Cox et al., 2005; Esterhuizen and Downing, 2008; Metcalf et al., 2008, Baptista et al., 2011, Cianca et al., 2012). However, many works reported BMAA not to

be detected (Krüger et al., 2010; Rosén and Hellenäs, 2008; Fan et al., 2015), detected in only a few samples (Faassen et al., 2009) or detected at residual concentrations (Jonasson et al., 2010; Li et al., 2010).

This suggested that, since cyanobacteria have a global distribution the occurrence of BMAA may be globally widespread, especially since blooms of cyanobacteria are becoming increasingly common.

BMAA presence in biological samples has been reported using different extraction and analytical methods (Faassen 2014). The existence of numerous isomers of BMAA constitutes a challenge when detecting this amino acid in biological samples. The risk of quantifying these isomers instead of BMAA has been addressed (Jiang et al. 2012).

Different isomers of BMAA have been reported such as DAB (2,4-diaminobutyric acid), AEG (N-(2-aminoethyl) glycine), and β-amino-*N*-methyl-alanine (BAMA) which are molecular isomers (Fig. 1). DAB is believed to be a hepatotoxic and neurotoxic non-proteinogenic amino acid, found in many prokaryotic and eukaryotic organisms (Jiang et al., 2012). AEG is a pillar for peptide nucleic acids and its effects, if any, have yet to be reported (Banack et al., 2012).

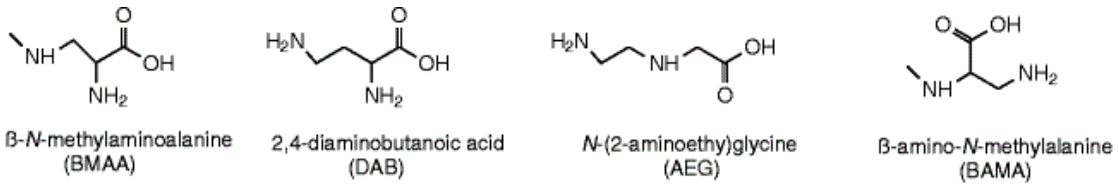

Fig. 1 - Structure of BMAA and selected isomeric compounds: DAB, AEG and BAMA (Adapted from Jiang et al., 2012).

Cohen (2012) has reviewed the analytical methods for detection and quantification of BMAA in biological samples, addressing sample preparation and the use (or not) of derivatization. Faassen et al. (2012) validated methods for BMAA determination in cyanobacteria samples by reversed-phase liquid chromatography with fluorescence detection (RPLC-FLD) and liquid chromatography – tandem mass spectrometry (LC-MS/MS) with the derivatizing agent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ®-Tag, Waters) and by hydrophilic interaction liquid chromatography (HILIC) LC-MS/MS without derivatization and concluded that the use of an analytical method with MS/MS detection is needed to try to prevent the incorrect identification or quantification of BMAA.

Faassen et al. (2012) suggested that the variation in results may to be related to the analytical method used. It has been also suggested that matrix effects may be complicating factors in BMAA analysis (Glover et al., 2012). BMAA is a highly reactive molecule and it may interact with other molecules during the analysis, which could prevent accurate quantification (Li et al., 2010; Glover et al., 2012). If the matrix is of higher complexity, other chemical interactions could interfere with the analysis (Li et al., 2010; Glover et al., 2012). Finally, the lack of a standard procedure to determine BMAA in various samples (e.g. cyanobacteria, marine invertebrates and vertebrates, etc.) constitutes a problem in the accurate determination of BMAA: Therefore, failure to detect BMAA cannot be taken as a total absence of the compound.

1.2.3. Biosynthesis

Variation of cellular toxin levels under different growth conditions have been studied for a number of toxin-producing cyanobacteria (Rapala and Sivonen, 1998, Sivonen and Jones, 1999). Some studies have established a relation between environmental conditions and cyanotoxin production (Rapala and Sivonen, 1998; Vézie et al., 2002; Jähnichen et al., 2007). Efforts rely on unveiling not only the mechanism that underlie toxin biosynthesis but also its role in cyanobacteria, which remains a mystery. Nonetheless, it remains a difficulty to identify all the factors involved in cyanotoxin biosynthesis, how these are regulated at a molecular level and how this translates to actual responses in the environment (Neilan et al., 2013). Some cyanotoxins are constitutively produced and potentially less dependent on the environmental conditions.

Some cyanotoxins, such as MC, NOD and CYN have a complete gene cluster for its biosynthesis characterized in different strains of cyanobacteria (Moffit and Neilan, 2004; Mbedi et al., 2005; Micallef et al., 2015). However, for the vast majority of the toxic secondary metabolites produced by cyanobacteria, a clearly identified biosynthesis pathway has not been put forward. Such is the case of BMAA.

The production of BMAA by cyanobacteria in culture media has been reported not to be consistent (Downing et al. 2011; Li et al. 2010), and since no pathway of BMAA synthesis has been described, the conditions that favour its production by cyanobacteria remain to be ascertained. Downing et al. (2011) suggested that BMAA would be synthesized as a response to nitrogen depletion in culture media. However, in other studies, this was not verified (Baptista et al., 2015). In fact, different strains of cyanobacteria appeared to respond better, to the presence of nitrogen in the medium regarding BMAA productivity.

1.2.4. Mechanisms of toxicity

Neurotoxic potential of BMAA is given by carbamate formation at both 3-methylamino group and 2-amino groups (Nunn and Ponnusamy, 2009), since the resulting molecule, a β-carbamate, has a structure similar to other excitatory amino acids (EAA) such as glutamate (Fig. 2)

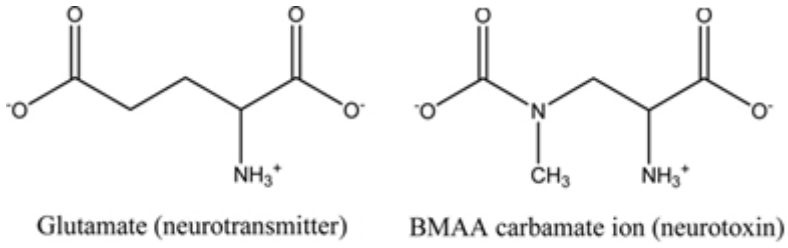

Fig. 2 - Structural similarities between the neurotransmitter L-glutamate and the carbamate adduct of BMAA. (From Erdner et al., 2008)

The neurotoxicity of BMAA is currently recognized on cellular and *in vivo* level (Chiu et al., 2011; Karamyan et al, 2008). Several toxicity mechanisms have been appointed as accountable for the connection between BMAA exposure and neurodegenerative diseases (Chiu et al., 2011; Okle et al., 2013). One of the mechanisms of action is excitotoxicity. Typically, this type of toxicity involves malfunctions in neurotransmitters within the nervous system, such as glutamate, which can ultimately lead to neuronal damage and degeneration (Chiu et al., 2011). Glutamate is one of the predominant EAA in the mammalian brain. In addition to its action as a synaptic neurotransmitter, it produces long-lasting changes in neuronal excitability, synaptic organization, neuronal migration during development, and neuronal viability (Meldrum, 2000; Tsien et al., 1996). The excitatory responses of this neurotransmitter are mediated by a number of distinct cell-membrane receptors, such as ionotropic glutamate receptors (iGluR). This type of glutamate receptors includes different classes like N-methyl-D-aspartate (NMDA), kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Michaelis, 1998).

Increased levels of glutamate were reported in the cerebrospinal fluid of ALS patients suggesting excitotoxicity may be involved in neurodegeneration (Majoor-Krakauer et al., 2003; Shaw, 2005). Moreover, studies have shown BMAA affects cultured neurons via mechanisms involving over-activation of iGluR, by mimicking the effect of glutamate naturally present in the brain. Also, low levels of BMAA were

associated with selective damage of sub-populations of neurons, such as motor neurons, via activation of AMPA/kainate receptors (Rao et al., 2006).

After consumption, BMAA passes from the gut into the blood stream and crosses the blood-brain barrier via large neutral amino acid carriers (Chiu et al., 2011). At this point, the formation of a carbamate adduct of the side-chain amino group produces structures capable of activating glutamate receptors by mimicking the effect of glutamate naturally present in the brain (Weiss et al, 1989; Rakonczay et al., 1991). The excess of BMAA leads to an overflow of calcium ions into the cells, and subsequent cell damage (Choi, 1988). The increased Ca^{2+} concentration in the cell causes the activation of lysozymes that inactivate the mitochondria by preventing the ATP synthesis associated with respiratory oxidation. Less ATP leads to less ATPases and Ca^{2+} enzymes, forming a vicious cycle with increased intracellular calcium, ultimately ending in cell death (Stout et al., 1998; Lobner et al., 2007; Chiu et al., 2011).

Excitotoxicity has been described for other amino acids such as β-N-oxalyl-amino-L-alanine (BOAA) and Domoic Acid (DA). BOAA can be found in the legume *Lathyrus sativus* and is responsible for the occurrence of Human Lathyrism (Spencer et al., 1987; Weiss et al.1989), by acting as a selective AMPA agonist. DA is a kainate agonist and its presence in algal blooms is associated with the occurrence of amnesic shellfish poisoning (ASP) (Wright et al., 1989; Todd, 1993). Activation of iGluR is also related to free radical production by calcium-dependent activation of the arachidonic acid cascade, nitric oxide synthase and calpain (Szydlowska and Tymianski, 2010). With time, reactive oxygen species can lead to irreversible cellular injury and death, by damaging proteins, lipids and DNA, and consequently leading to function impairment of vital macromolecules and organelles. BMAA is also responsible for the inhibition of the cysteine/glutamate antiporter system Xc-. Thus, the uptake of cysteine is blocked, which leads to glutathione depletion, and ultimately results in oxidative stress increase. Simultaneously, the system Xc- potentiates the release of glutamate from the cell. By binding to iGluR, excess glutamate induces further neurological damage by excitotoxicity (Liu et al., 2009). Despite the information regarding BMAA effects in neuronal cells the, effects upon iGluR have not been yet tested in marine organisms.

Recent studies appoint BMAA as capable of being incorporated into proteins and subsequently lead to protein misfolding (Banack et al., 2010). The tRNA synthetase enzyme for the amino acid serine mistakenly picks up BMAA and incorporates it into proteins *in vitro*. Amino acid misincorporation leads to mistakes in translation, exposing the hydrophobic parts of the protein. The resulting misfolded proteins start to “aggregate” forming larger clusters, until the cells are no longer able to function effectively (Dunlop et al., 2013).

1.3. Health and environmental risks of exposure to BMAA

1.3.1. Biomagnification hypothesis and human exposure

The bioaccumulation of cyanotoxins by aquatic organisms is widely described in literature. Several studies have shown that mollusks are among the most harmed organisms, being able to accumulate several cyanotoxins, such as MC (Amorim and Vasconcelos, 1999, Pires et al., 2004; Paldavičienė, et al., 2015), NOD (Sipiä, et al., 2002; Karlsson et al., 2003), paralytic shellfish poisoning toxins (Pereira et al., 2004; Setälä et al., 2014), CYN (Saker et al., 2004), anatoxin-a (Osswald et al., 2008) and okadaic acid (Silva et al., 2013; Garcia et al., 2015). More recently, the presence of BMAA in food webs of the North Atlantic was verified, suggesting that certain diets and locations may put people at particular risk (Jonasson et al., 2010; Brand et al., 2010). In the Baltic Sea, BMAA was found in organisms of higher trophic levels such as various vertebrates and invertebrates such as mussels and oysters (Jonasson et al., 2010). Also, in a recent long term monitoring of BMAA in cyanobacteria, mollusks, crustaceans and various fish species at different trophic levels in Gonghu Bay, bioaccumulation of the toxin was observed among the aquatic animals, and within the food web (Jiao et al., 2014). Over 7 months of study, BMAA content in cyanobacteria, mollusks, crustaceans and various fish species averaged between 4.12 – 6.05 μg BMAA g^{-1} dry weight (dw). The transfer and bioaccumulation of BMAA within these and other food webs illuminate the possible pathways of human exposure.

Mytilus galloprovincialis is a mollusk native to the Mediterranean coast and the Black and Adriatic Seas. It has succeeded in establishing itself at widely distributed points around the globe, occurring essentially in temperate regions, including the Atlantic Ocean (Branch and Stephanni, 2004). It is a smooth-shelled mussel with coloring usually ranging from blue-violet to black. The two shells are of quadrangular shape and equal dimensions. Generally, on one side, the edge of the shell ends with a pointed and slightly bent umbo while the other side is rounder. This animal can grow up to 140 mm in length and it can inhabit from exposed rocky outer coasts to sandy bottoms (Ceccherelli and Rossi 1984). *M. galloprovincialis* constitutes an important component of estuarine and marine food webs and because of its sessile filter feeder nature, it may be exposed to high density of cyanobacteria and their toxins (Osswald et al., 2008). This species is eaten by other animals (including humans), providing a way

of exposure to cyanotoxins, namely BMAA, through food chain. In fact, it was recently shown that BMAA is accumulated in this organism (Baptista et al., 2015).

Thus human exposure to BMAA may come from direct contact with cyanobacterial blooms or consumption of aquatic organisms exposed to such blooms (Jonasson et al. 2010; Cox et al., 2005). This suggests that BMAA, like cyanobacteria, may have a global distribution and therefore the occurrence of BMAA may be a worldwide concern.

Due to this fact, and to the discrepancies documented on BMAA measurement in environmental samples (Banack et al., 2010; Faassen, 2014; Glover et al., 2012) the assessment of this amino acid in the aquatic environment, should take into account not only its quantification, but also its effects towards aquatic organisms.

1.3.2. Biomarkers of toxic exposure

Biomarkers are helpful tools in detecting and evaluating the harmful consequences of chemical contamination on organisms. They can be subdivided into three classes (NRC, 1987; WHO, 1993). However, one biomarker can be classified within several groups

Table 1 – Biomarker Classification according to NRC (1987) and WHO (1993).

Biomarker Classification	**Description**
Exposure	used for confirmation and assessment of exposure to the toxicant
Effect	allow the establishment of negative effects caused by exposure to the toxicant
Susceptibility	elucidate on the variations in the response sensibility to toxicant exposure between different individuals

The subdivision of biomarkers in the literature is somewhat diffuse since biomarkers of exposure and those of effect are actually distinguished by the way they are used (van der Oost et al., 2003)

It is important to note that despite its classification, an individual biomarker is not able to reflect the status of the organism's health and the effects of each toxicant in a mixture. Moreover, when using biomarkers for in-field studies, results are often difficult to interpret due to the influence of environmental variables and the exposure of

the studied organisms to a mixture of several chemicals with influence on the measured molecular and/or cellular responses (Frasco et al., 2005). For these reasons, the use of a biomarker battery is recommended when assessing the biological impact of chemical pollutants (Regoli et al., 2004).

Biomarkers often provide faster biological answers and information about toxicant bioavailability in comparison with other toxicological methods or assays (Shugart et al. 1992). An accurate prediction of effects at higher levels of organization in the ecosystem makes biochemical changes useful biomarkers. These variations allow the assessment of whether toxicants are present, as well as the natural response of a particular organism to a specific environmental stressor. Regarding cyanobacterial exposure, parameters of biotransformation or oxidative stress are usually addressed (Bláha et al. 2004; Ziková et al., 2013; Guzmán-Guillén et al., 2014).

Biomarkers are common in the biomonitoring of contaminated marine environments. Typically, they rely on the study of potential stimulation or inhibition of specific enzymes, such as Glutathione s-tranferases (GSTs) and Acetylcholinesterase (AChE) (Moreira and Guilhermino, 2005). GSTs are a family of phase II dimeric enzymes involved in the detoxification of xenobiotics with an electrophilic center by conjugating them with reduced glutathione (GSH) (Hayes et al., 2005). The activity of these detoxification enzymes has been successfully used as a biomarker of exposure to toxic compounds in bivalves (Sheehan and Power, 1999). Increased GSTs activity has been reported, not only in mussels but also in various other aquatic organisms (Pflugmacher et al., 1998) in connection to exposure to cyanobacterial toxins, such as MC and CYN (Pflugmacher et al., 1998; Wiegand et al.,1999; Nogueira et al., 2004; Sipiä et al., 2002, Vasconcelos et al., 2007). AChE is an indicator of neurotoxic effects (Kirby et al., 2000) and has traditionally been used as a biomarker of exposure to organophosphate and carbamate pesticides (Bocquené and Galgani, 1998). It is usually a general indicator of pollution (Schiedek et al., 2006), and recently was also proven to be affected by exposure to NOD (Lehtonen et al., 2003), as well as other cyanotoxins (Ferrão-Filho and Kozlowsky-Suzuki, 2011).

More recently, research efforts have been converging on particular genes sequencing and transcription to be used as potential biomarkers. In fact, this molecular biology approach (transcriptomics) has been already used in the biomonitoring of contaminated sites (Hoarau et al., 2006; Sarkar et al, 2006). Gene expression biomarkers can enable rapid assessment of physiological conditions *in situ*, providing a valuable tool for linking organism physiology with cyanobacterial toxic exposure. In most studies, there is a focus on the genes which are either involved in the metabolic activation and detoxification of xenobiotics, or in oxidative stress regulation (Kurelec et

al., 1996; Blanchette et al., 2007; Izagirre et al, 2014). However, it has become increasingly clear with time that single biomarkers are not able to describe the physiological status of the mussel. Instead, the use of a battery of biomarkers is becoming a standard (Narbonne et al., 2005; Cotou et al., 2013; Izagirre et al, 2014).

1.4. Objectives

In marine environments BMAA has been shown to find its way from the phytoplankton first producers (e.g. cyanobacteria) to marine invertebrates first consumers such as *M. galloprovincialis*, which accumulate this amino acid. Therefore, the aim of this work was to assess the use of the activity of the enzymes GSTs and AChE, and the expression of iGluR genes, as biomarkers of BMAA exposure in *M. galloprovincialis*.

To this aim, genetic expression of two iGluR genes was evaluated by a quantitative polymerase chain reaction (qPCR), and GSTs and AChE activity was measured spectrophotometrically in gills and digestive gland of *M. galloprovincialis* exposed to BMAA standard, or fed with BMAA-producing cyanobacterium.

BMAA effects upon enzyme activity, in *M. galloprovincialis*, have not been tested yet, despite the fact that these are commonly assessed biomarkers for exposure to cyanotoxins.

Effects upon glutamate receptors have also not been yet tested in *M. galloprovincialis*. In fact, the presence of iGluR in *M. galloprovincialis* has only been putatively ascertained, through comparison with iGluR sequences from other organisms. Therefore, the first part of this analysis consisted in determining iGluR sequences of interest and characterizing them with the support of bioinformatics tools.

2. Material & Methods

2.1. Mussel collection

Collection of *M. galloprovincialis* took place in a beach at Matosinhos, Portugal, in October 2014. The mussels were placed in aquariums at BOGA (Biotério de organismos aquáticos) at CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental), for acclimation, during 3 days. The aquariums used in this experiment had capacity of 5 L, were filled with 3 L of filtered sea water, aerated and maintained at 18ºC.

Collection of mussels was performed in Italy. *M. galloprovincialis* were brought from a lagoon outside of Venice, in February 2015. They were maintained in aquariums with 14 L of capacity, filled with 10 L aerated sea water at 14°C for 5 days for acclimation.

In both cases the mussels were not fed. Water was renewed every two days.

2.2. Bioaccumulation experiment

2.2.1. Mussels exposed to BMAA standard

For analysis of AChE and GSTs activity *M. galloprovincialis*, collected at Portugal, at densities of 24 individuals per tank, were exposed to BMAA standard (Sigma–Aldrich) with concentrations of 10 µg L^{-1}, 100 µg L^{-1}, and 1000 µg L^{-1} dissolved in filtered seawater for a period of 48h. All experiments were conducted in triplicate. Control mussels (not exposed to BMAA) were kept likewise in filtered seawater. Four individuals were collected from the BMAA treatment and control aquariums at 12h, 24h and 48h. At this point the water was changed and mussels depurated for 2 days. During this period, at 72h and 96h, 4 individuals were also collected from the BMAA treatment and control aquariums. The mussels were not fed, and no deaths were observed during this experiment. Upon collection, mussels were dissected and separated in gills and digestive gland. Tissues were then frozen at -80ºC.

For analysis of iGluR expression, mussels collected at Italy, at densities of 24 individuals per tank, were exposed to 1000 ug L^{-1} standard BMAA dissolved in seawater for a period of 48h. Four individuals were collected from the BMAA treatment and control aquariums at 6h, 24h, 48h. At this point the water was changed and

mussels depurated for 1 day. At 72h 4 individuals were also collected from the BMAA treatment and control aquariums. The mussels were not fed. Five deaths were observed in the control aquarium. Five deaths were also observed in the aquarium treated with BMAA during this experiment.

Upon collection, mussel sex and gonad status was registered. A mix of females and males in similar conditions were used, to avoid variability induced by discrepancies in reproductive stage. Mussels were dissected and separated in gills and digestive gland. Tissues were then frozen at -80ºC.

2.2.2. Mussels fed with cyanobacteria

Animals were kept in 0.5 L aquariums of filtered seawater, with aeration and natural light, at 18 °C. After an acclimation period of 3 days, mussels in each aquarium were fed either with cyanobacteria *Nostoc* sp. (LEGE 06077), or *Microcoleus* sp. (LEGE 07092), from the LEGE culture collection, or with the green algae *Chlorella* sp. (LEGE Z-001). Cyanobacteria sequences associated with this study are available in GenBank under the accession numbers HM217071 and HM217062, respectively.

Cyanobacteria species were grown in BG11 culture medium (Rippka et al., 1979), with a light intensity of 25 µE $m^{-2}s^{-1}$ and a light:dark period of 14h:10h, at 25ºC. *Chlorella* sp. was grown in Z8 culture medium (Kotai, 1972) in the same conditions described above.

Mussels were fed once each day with approximately 10^5 cells mL^{-1} of either *Nostoc* sp., *Microcoleus* sp or *Chlorella* sp. From each aquarium, 4 mussels were retrieved, at 24h and 48h. Upon collection, mussels were dissected and gills and digestive gland were separated for posterior analysis of enzymatic activity. Tissues were immediately frozen at -80ºC awaiting further procedures.

2.3. Enzymatic analysis

2.3.1. Tissue homogenization and protein quantification

Approximately 250 mg of tissue from digestive gland or from gills were homogenized with liquid nitrogen and a protein solubilization buffer (SB). SB was prepared as a solution of phosphate buffer (100 mM, pH 7.0) containing 20% glycerol (v/v), 1.4 mM dithiothreitol (DTT) and 1 mM Ethylenediaminetetraacetic acid (EDTA) to aid in the extraction and stabilization of proteins present in the mussel tissues.

Homogenization was made maintaining a constant ratio of 5 mL of buffer for each gram of tissue. Cell debris were ultra-centrifuged (Beckman Coulter centrifuge, model Alegra 25R, Beckman Coulter, CA, USA) at 100 000 ×*g* for 60 min, at 4ºC. The supernatants were stored at −80 °C for posterior analysis.

Total protein quantification was conducted using a microplate-adapted protocol of the Bradford method (Bradford, 1976). The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change.

A standard curve was prepared using standards containing a range of 0.25 mg mL^{-1} to 1.25 mg mL^{-1} bovine serum albumin. In the microplate, 5 µL of standard or sample were added to the wells. After addition of 250 µL dye reagent to the wells and an incubation period of 15 min absorbance measurements were performed at 595 nm. Protein quantification was obtained by comparison with standard curve previously obtained. Sample protein content was then adjusted to 0.3 mg mL^{-1}.

2.3.2. GSTs activity quantification

GSTs activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB), which is suitable for the broadest range of GST isoenzymes. The reaction is based on the principle that upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance at 340 nm. Procedures were as described by Habig, (1974) adapted to microplate, with alterations from Frasco and Guilhermino, (2002).

To obtain the reaction mixture, CDNB (60 mM) was dissolved in ethanol, with the final reaction concentration less than 0.01%, and GSH (10 mM) was dissolved in phosphate buffer (100 mM, pH 7.0).

In 96-well microplates, 0.2 mL of the reaction mixture was added to 0.1 mL of the sample and the GSTs activity was measured immediately every 20 seconds, at 340 nm, during 5 minutes, at 25ºC, in triplicate on Bioteck microplate reader (Synergy HT, 2009).

2.3.3. AChE activity quantification

AChE activity was measured using DTNB (acid dithiobisnitobenzoate) to quantify the thiocholine produced from the hydrolysis of acetylthiocholine by AChE. The

absorption intensity of DTNB adduct is used to measure the amount of thiocholine formed, which is proportional to the AChE activity. The method of Ellman et al. (1961), adapted to microplate, was followed.

In the assay, 0.250 mL of the reaction solution (30 mL of phosphate buffer, 1 mL of 10 mM DTNB (acid dithiobisnitobenzoate mixed with sodium hydrogen carbonate in phosphate buffer) and 0.200 mL of acetylthiocholine iodide 0.075 M) were added to 0.05 mL of homogenized sample tissue. The measurements were performed after an incubation period of 5 min, at 412 nm, during 10 minutes, every 15 seconds, at 25ºC, in triplicate on Bioteck microplate reader (Synergy HT, 2009).

2.4. iGluR expression

2.4.1. Sequence selection and preliminary analysis

A search in National Center for Biotechnology Information (NCBI) databases showed that there were no available sequences for glutamate receptors in *M. galloprovincialis*, or in *Mytilus* sp. in general. Therefore, the first part of this work was devoted to finding sequences that could putatively be considered as glutamate receptors, in *M. galloprovincialis*.

For that purpose, a catalogue of ESTs from *M. galloprovincialis* named Mytibase was used (Venier et al., 2009), at the Department of Biology of University of Padova. Seven putative glutamate receptor sequences were initially retrieved. Nucleotide and deduced amino acid sequences were obtained, after similarity to orthologue sequences was determined.

Digital gene expression was performed with CLC Genomics Workbench to ensure sequences with higher expression values in *M. galloprovincialis* tissues were selected (Annex 1). Based on this preliminary analysis, two sequences, termed GLU4 and GLU5 were chosen for further work.

iGluR all share a common membrane topology (Fig. 3) characterized by a large extracellular N-terminus that includes the N-terminal domain (S1), a membrane region comprising three transmembrane segments (M1, M3 and M4,) plus a re-entrant pore loop (M2,), an extracellular loop between M3 and M4 where the ligand-binding domain (S2) is located, and a cytoplasmic C-terminus, which varies in size and provides multiple sites of interaction with numerous intracellular proteins (Wood et al., 1995; Dingledine et al., 1999).

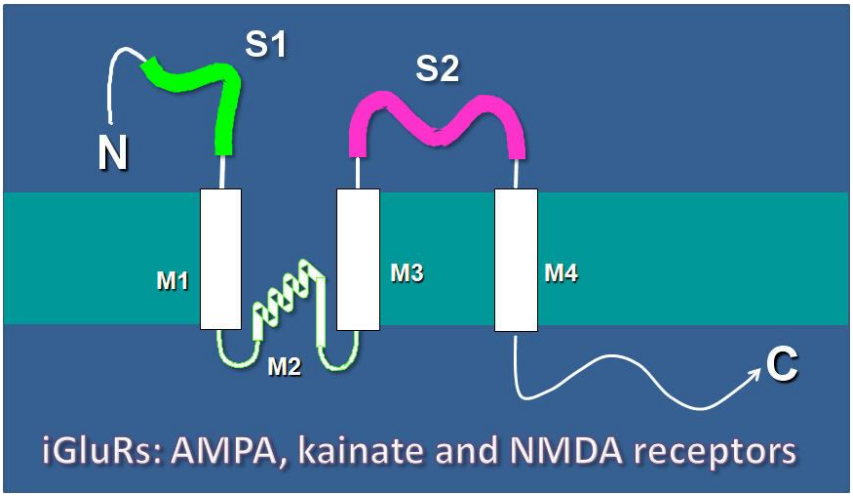

Fig. 3 - Membrane topology of iGluR. S1 – N-terminal domain; S2 – Ligand-binding region; M1, M3 and M4 – Transmembrane domain; M2 – Re-entrant pore loop (From VanDongen Lab: http://people.duke.edu/~av8/vandongen_lab/Research).

After selection of GLU4 and GLU5, further work was carried out to characterize the sequences. In order to have an idea of GLU4 and GLU5 domain architectures SMART was used (Schultz et al., 1998; Letunic et al., 2015). The presence of signature domains consistent with iGluR receptors, such as L-glutamate binding region was observed (Annex 2). This online tool also allowed the retrieval of several sequences from different *taxa* with domain organization similar to GLU4 and GLU5. Afterwards, all sequences were aligned using the Clustal Omega (Sievers et al., 2011). After alignment (Annex 3), a phylogenetic tree was generated with Phylogeny.fr platform (Dereeper et al., 2008) to analyze relationships between mussel and other *taxa* iGluR sequences (Annex 4).

Hydropathic analysis with Phobius (Käll et al., 2004) of the predicted mature polypeptides of GLU4 and GLU5 showed the presence of three strongly hydrophobic regions (Annex 5 and 6; images obtained with Protter (Omasits et al. 2013)). The N termini of GLU4 and GLU5 contained signal peptides of 20 and 23 amino acids, respectively. The distribution of hydrophobic regions and potential sites for N-glycosylation suggests that the topology of GLU4 and GLU5 is similar to that predicted for the mammalian iGluR subunits. The present sequences, however, appear to lack N-terminal domain present typically in iGluR (Annex 2).

2.4.2. Primer design

Open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/projects/gorf/)

was used to identify all open reading frames using the standard or alternative genetic codes. The coding regions of GLU4 and GLU5 are of 1467 and 1338 nucleotides long, respectively. Primers were designed with Primer 3 (Untergrasser et al., 2012; Koressaar and Remm, 2007), to fit in the selected ORF (Annex 7) for each sequence and in accordance to the following guidelines: product size 100-250 bp, melting temperatures Tm 60±1 °C, and G/C ≤50 %. Primer details are reported in Table 2. The housekeeping gene Elongation factor 1 alpha (EF-1 α) was chosen as internal reference because the level of RNA remains essentially constant from sample to sample and the primers have been used as described in Gerdol et al. (2011).

Table 2 - Primers designed for analysis of GLU4 and GLU5 sequences and the housekeeping gene EF-1 α

Primer name	**Description**	**Sequence 5'-3'**	**Amplicon size (bp)**
GLU4	Glutamate receptor	F:5'-GTGAGCCATTTCTGCTCCAG-3' R: 5'-GATGGATCGTTTGCAGCCAT-3'	212
GLU5	Glutamate receptor	F:5'-GATGCCAAAGACCGTAGCTG-3' R:5'-TTCCAATCCACACGCAAAGG-3'	175
EF-1 α	Elongation factor	F:5'-CCTCCCACCATCAAGACCTA-3' R:5'-GGCTGGAGCAAAGGTAACAA-3'	130

Primer specificity and the presence of an unique amplicon was verified by blasting primers sequences against mussel cDNA library Mytibase.

2.4.3. RNA extraction and purification

Total RNA from approximately 100 mg of frozen gill or digestive gland was extracted using Trizol reagent (Invitrogen) following the manufacturer's recommendations. Following isolation of total RNA, the RNA was further cleaned by LiCl (8 M) purification. Pellets were washed twice with 75% ethanol (EtOH) and resuspended in 200 µL of RNAse free water.

For RNA quantification a ND-1000 UV/visible spectrometer (NanoDrop Technologies) was used, with a sample volume of 1 µL. In the samples RNA concentration ranged between 233 and 1914 ng μL^{-1} (Table 3). Samples were

considered pure regarding contamination from protein and carbohydrates, since the values of the ratio of the wavelengths 260/280 and 260/230 were above 2.0 and 2.2, respectively.

Table 3 – Nanodrop quantification of RNA concentration in exposed digestive gland and gill tissues and paired controls. Absorbance spectroscopy gives a concentration of RNA in ng μL^{-1}.

Organ	Time (h)	Treatment	RNA (ng μL^{-1})
Digestive Gland	6	Control	1164
Digestive Gland	24	Control	1355
Digestive Gland	48	Control	1324
Digestive Gland	72	Control	1353
Digestive Gland	6	BMAA	1568
Digestive Gland	24	BMAA	1390
Digestive Gland	48	BMAA	1866
Digestive Gland	72	BMAA	1914
Gills	6	Control	434
Gills	24	Control	366
Gills	48	Control	366
Gills	72	Control	362
Gills	6	BMAA	864
Gills	24	BMAA	404
Gills	48	BMAA	321
Gills	72	BMAA	233

The qualitative analysis of RNA was performed on gill and digestive gland RNA samples using the RNA 6000 Nano LabChip kit (Agilent Technologies) in association with the Agilent 2100 Bioanalyzer.

The RNA Gel Matrix, provided with the kit, was filtered through spin columns (Costar) and then centrifuged at 1500 x *g*, during 10 min at room temperature. In the chip, 32.5 mL of filtered Gel Matrix, plus 0.5 µL of dye concentrate were charged. The samples were then loaded into the wells, and the markers (RNA 6000 Nano Marker, Agilent) were also loaded. Finally, all wells were loaded with 1 mL of RNA 6000 Ladder-Ambion previously denatured. The instrument sequentially ran the ladder and samples

quantitating the fluorescence emitted by a red fluorescent intercalating dye as the RNA passed a fixed point within the capillary.

Figure 4 shows some of the electrophoregrams obtained showing that the quality of the extracted RNA was suitable for the analysis of GLU4 and GLU5. Electropherograms were obtained with Agilent 2100 Bioanalyzer, for *M. galloprovincialis* gill and digestive gland total RNA.

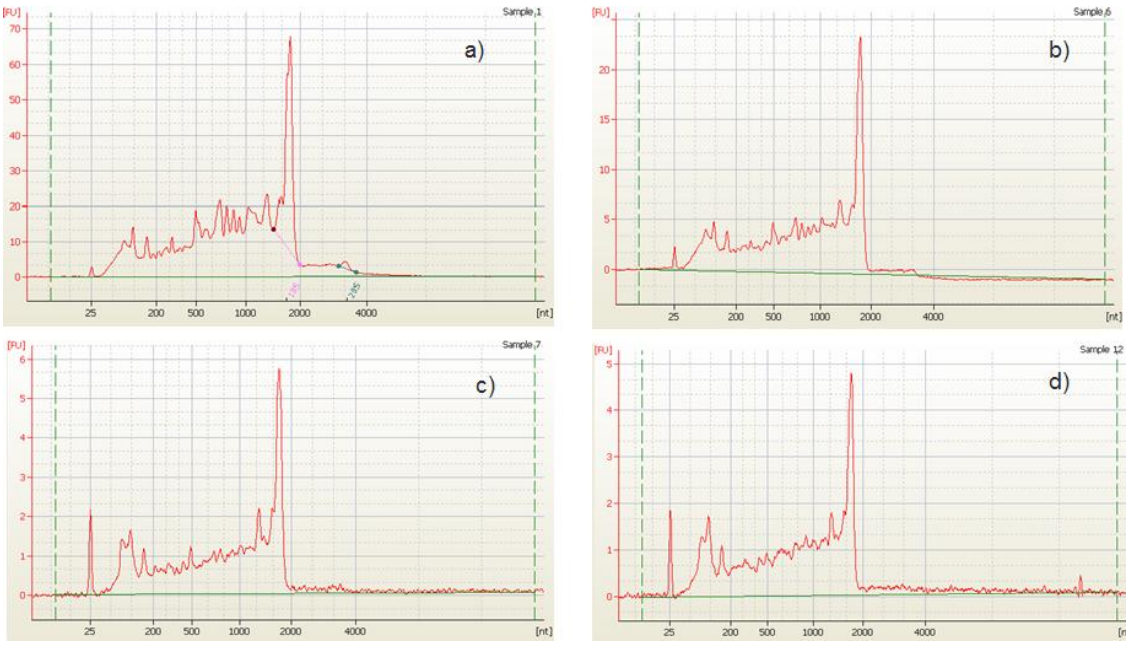

Fig. 4 - Electropherogram (from the Agilent 2100 Bioanalyzer) for *M. galloprovincialis* gill and digestive gland total RNA. The x-axis represents RNA length in nucleotides (nt), and fluorescence (FU) is represented on the y-axis. The quality was ascertained by RNA integrity number (RIN). This algorithm divides the RNA profile into nine different regions and applies a continuous value from 10 to 1 defining the extent of RNA degradation, 10 being the highest quality. All images shown represent samples with RIN above 6: a) control digestive gland at 6h; b) exposed digestive gland at 72h; c) Control gills at 6h; d) exposed gills at 72h.

2.4.4. Quantitative PCR for gene expression analysis

The expression levels of GLU4 and GLU5, were assessed in samples of the digestive gland and gills, of four adult mussels, collected at each time point, from the control (0 µg L^{-1}) and the BMAA treated (1000 µg L^{-1}) aquariums.

RNA pools were prepared with equal amounts of RNA from each individual mussel (N = 4). The cDNA for qPCR was obtained using a Superscript II Reverse Transcriptase 1st Strand cDNA Kit (Invitrogen) from 1 µg total RNA. PCR reactions were performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using DyNAmo HS SYBR Green qPCR kit (Thermo Scientific) to amplify 1 µL of

purified first-strand cDNA in a 10 µL of final reaction mixture.

Thermal cycling conditions were: 15 min denaturation at 95ºC; followed by 40 cycles of 30 s denaturation step at 95ºC, annealing and elongation steps for 1 min each at 60ºC. A dissociation curve analysis was performed at the end of the reaction to ensure the specificity of the primers. Three replicates were amplified of the complete sample set (BMAA exposed mussels and paired controls, 4 time points) for each primer pair (target and endogenous genes). The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. To calculate the relative expression ratio, the $2^{-\Delta\Delta Ct}$ (RQ, relative quantification) method (Livak and Schmittgen, 2001) implemented in the 7500 Real-Time PCR System software was used.

Additionally, the primers were subjected to a preliminary test with spring mussel gill samples, in order to confirm the presence of unique amplicons and exclude the possibility of primer dimers. The gill samples for this evaluation originated from mussels collected from an outlet of the Venice lagoon (Italy) in May 2014, and acclimated at 23ºC and 32 ‰ salinity. After 24h and 48h the mussels were retrieved and processed as described before for qPCR. Dissociation curves of the qPCR products for both GLU4 and GLU5 transcripts using these mussels showed single peaks.

2.5. BMAA quantification

2.5.1. Microwave-assisted digestion

Soft tissues from mussels exposed to 10 µg L^{-1}, 100 µg L^{-1} and 1000 µg L^{-1} of BMAA standard, or fed with cyanobacteria or microalgae, were lyophilized before digestion (FTS System freeze dryer EZ550). Cyanobacteria and *Chlorella* sp. cultures used for feeding were harvested by centrifugation and also lyophilized.

In both cases, approximately 10 mg (dw) were acid-digested with 2 mL of 6M of HCl, at 120ºC, for 20 minutes using a high-pressure microwave system (Milestone-Ethos 1). The samples were evaporated in a low flux of nitrogen and then reconstituted in 0.5 mL 20 mmol L^{-1} HCl, prior to analysis by LC-MS/MS.

2.5.2. Liquid chromatography with mass detection (LC-MS/MS) analysis.

The analyses were performed in a Thermo LCQ Fleet Ion Trap LC/MS^n system

(Thermo Scientific), using a 2.1 x 100 mm, 5 µm diameter ZIC-HILIC column (SeQuant), and a 14 x 1 mm, 5 µm guard column (SeQuant). The mobile phase consisted in eluent A, acetonitrile (0.1% formic acid) and eluent B, deionized water (0.1% formic acid). In the first 20 min a 90–60% linear gradient of acetonitrile was achieved, and afterwards 60% acetonitrile was maintained for 15 min. The system was then equilibrated to the initial conditions during 5 min (Kubo et al. 2008). The flow rate was 0.5 mL min^{-1}, the injection volume was 10 µL, and the column temperature was 40 ºC. The electrospray ionization (ESI) was operated in the positive mode. Nitrogen was used as sheath gas, at a rate of 45 (unitless), and auxiliary gas at a rate of 20 (unitless). The capillary temperature was held at 250 ºC.

Mass-to-charge ratio (*m/z*) scan was performed from 50 to 150, and the ion m/z 119 was monitored. At collision energy of 14 V the presence of more abundant product ions *m/z* 102, 88 and 76 was verified, in this order of abundance, as reported before (Rosén and Hellenäs, 2008) and selected-reaction monitoring (SRM) chromatograms were retrieved. *m/z* 102 was used to quantitatively assess BMAA and *m/z* 88 and *m/z* 76 used to qualitatively assess BMAA. SoftwareXcalibur® was used to analyze the data.

To account for matrix effects a calibration curve was prepared as described in Baptista et al. (2015). Mussel and cyanobacteria (10 mg dw) were spiked with BMAA standard in the 10 to 1000 µg L^{-1} range, and digested as described above. The limit of quantification (LOQ) was obtained from the calibration curve, calculated as 10 α /S, where α is the standard deviation of the y-intercept and S is the slope of regression line.

2.6. Data treatment

GSTs and AChE activity results are presented as mean values ± standard error of the mean (SEM) and were analyzed by one-way ANOVA. Post-hoc comparisons were made using Tukey's test considering $p \leq 0.05$ as statistically significant. Individual gene expression levels, of mussels exposed to BMAA and controls, were compared by using an unpaired Student's *t* test. GraphPad Prism 6 was used for the calculations.

3. Results

3.1. BMAA quantification in mussels and cyanobacteria

Mussels exposed to 1000 µg L^{-1} for 24h and 48h, presented detectable amounts of BMAA (Table 4). At this concentration, accumulation of BMAA by mussels was shown to be similar to previous exposures of *M. galloprovincialis* to the same range of concentrations (Baptista et al., 2015). No mortality was registered during the experiment, implying that acute lethal toxicity does not occur at the tested concentrations. In mussels not exposed (control), the amino acid was not detected. For 10 µg L^{-1} and 100 µg L^{-1} quantification was not possible due to lack of availability of the equipment.

Table 4 - Concentration of BMAA (µg g^{-1}) on mussels exposed to 1000 µg L^{-1} for 24 h and 48 h. Mean ± SEM for the concentration of BMAA.

Concentration tested	Exposure Time (h)	BMAA (µg g^{-1})
1000 µg L^{-1}	24	41.59 ± 4,27
	48	52.19 ± 4,43
0 µg L^{-1}	24	< LOQ
	48	< LOQ

Limit of quantification: LOQ = 0.8 µg g^{-1}

BMAA was analysed in the cyanobacteria and *Chlorella* sp. used for feeding the mussels. In *Nostoc* sp. BMAA could be quantified but in *Microcoleus* sp, no BMAA was detected (Table 5). For the microalgae *Chlorella* sp. no BMAA could be detected either.

Quantification of BMAA in the mussel fed with cyanobacteria and green alga was not possible. However, in a previous study, mussels fed with cyanobacteria *Synechocystis salina* showed accumulation of 32 µg g^{-1} of BMAA after 4 days of feeding (Baptista et al., 2015). In this study, one of the strains used, *Nostoc* sp., presented measurable levels of BMAA (Table 5). Given the levels of biomass used to feed the mussels in the accumulation experiment, these results suggest that *M. galloprovincialis* fed with *Nostoc* sp. were exposed to approximately 2 µg L^{-1} BMAA.

Since similar conditions of feeding were provided, it was assumed that mussels in the present work could also have accumulated BMAA after a 48h period.

Table 5 - BMAA quantification (µg g^{-1}) in the phytoplankton used to feed *M. galloprovincialis* and the culture media in which the phytoplankton was grown.

Strain	Culture medium	BMAA (µg g^{-1})
Nostoc sp.	BG11	2.65
Microcoleus sp	BG11	<LOQ
Chlorella sp.	Z8	<LOQ

3.2. AChE and GSTs analysis

3.2.1. Mussels exposed to BMAA standard

3.2.1.1. Digestive gland

During the exposure period the digestive glands of animals exposed to BMAA presented a significant increase in GSTs activity for the concentrations of 100 µg L^{-1} and 1000 µg L^{-1} ($p<0.05$) when compared to the control group (Fig. 5A). After 24h of exposure, GSTs activity increased significantly also in the digestive gland of mussels exposed to the 10 µg L^{-1} of BMAA ($p<0.05$).

During the depuration, the activity decreased returning to levels similar to the ones observed in control mussels (Fig. 5A).

For the AChE activity, no significant differences in digestive gland were found between the treated mussels and the control group throughout the experiment (Fig. 5B).

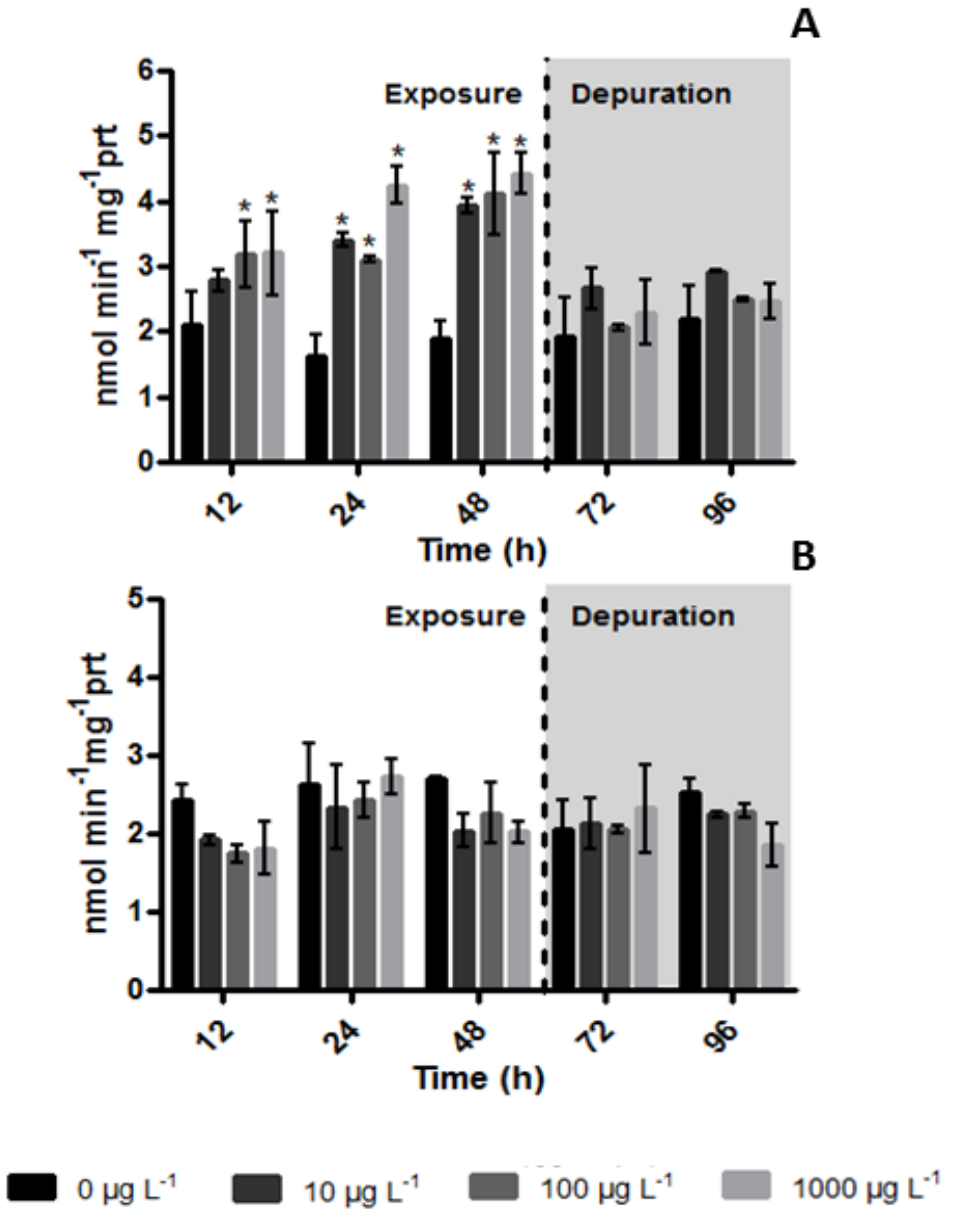

Fig. 5 - (A) GSTs activity and (B) AChE activity in *M. galloprovincialis* digestive gland exposed to BMAA standard over a period of 48h and depurated until 96h. Black columns represent Control group, dark grey columns represents group exposed to 10 µg L^{-1} BMAA, medium grey columns represent group exposed to 100 µg L^{-1} BMAA and light grey columns represent group exposed to1000 µg L^{-1} BMAA. Bars are the SEM of three replicates (each replicate comprising four individuals of *M. galloprovincialis*). Statistically significant differences were accepted at $p \leq 0.05$; * indicates differences to control.

3.3.1.2. Gills

GSTs activity was significantly higher in the gills of mussels exposed to 1000 µg L^{-1} ($p<0.05$), for 12h and 24h, compared to the control group. However, at 48h of exposure, levels of activity decrease and were no longer different from the control group.

During depuration, at 72h and 96h, the activity was significantly higher in the mussels exposed to 100 µg L^{-1} and 1000 µg L^{-1} ($p<0.05$) in comparison to the control group. For the lowest concentration, BMAA did not produce any significant effect in GSTs activity, as there were no differences between exposed and control mussels (Fig. 6A).

A significant decrease in gill AChE activity was verified between the treated and the control groups at 48h of exposure to BMAA, for 100 µg L^{-1} and 1000 µg L^{-1} concentrations ($p<0.05$) (Fig. 6B). During depuration, activity levels raised for these concentrations, returning to levels similar to the control group. At a concentration of 10 µg L^{-1}, no significant alterations were observed in gill AChE activity of *M. galloprovincialis* during the whole experiment.

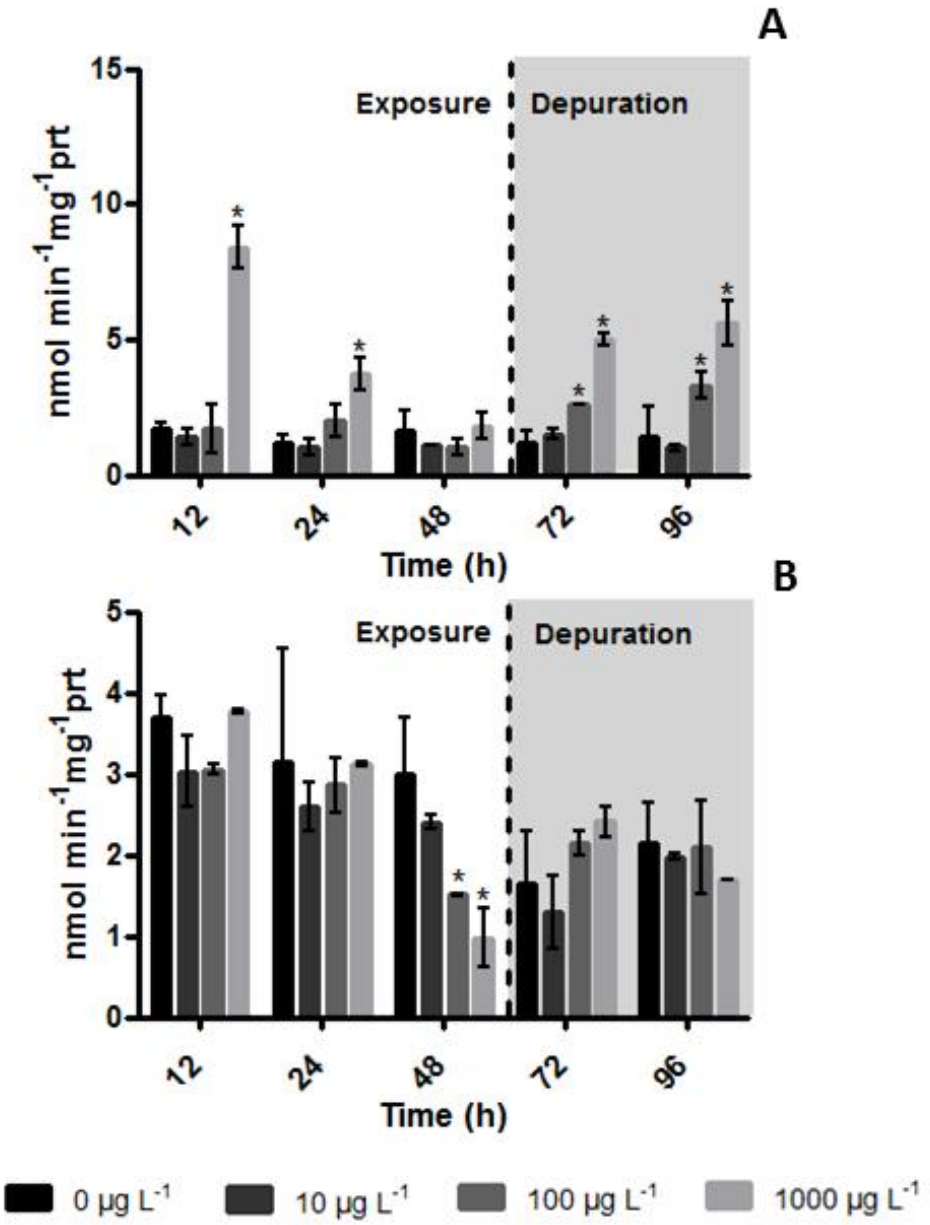

Fig. 6 - (A) GSTs activity and (B) AChE activity in *M. galloprovincialis* gills exposed to BMAA standard over a period of 48h and depurated until 96h. Black columns represent Control group, dark grey columns represents group exposed to 10 µg L^{-1} BMAA, medium grey columns represent group exposed to 100 µg L^{-1} BMAA and light grey columns represent group exposed to1000 µg L^{-1} BMAA. Bars are the SEM of three replicates (each replicate comprising four individuals of *M. galloprovincialis*).. Statistically significant differences were accepted at $p \leq 0.05$; * indicates differences to control.

3.3.2. Mussels fed with cyanobacteria

3.3.2.1. Digestive gland

Activities of GSTs and AChE in the digestive gland were significantly higher in mussels fed with *Nostoc* sp. ($p<0.05$), than in mussels fed with *Microcoleus* sp or *Chlorella* sp. after 24h and 48h of exposure (Fig. 7). Mussels fed with *Microcoleus* sp or *Chlorella* sp. did not exhibited different levels of enzymatic activity (Fig. 7).

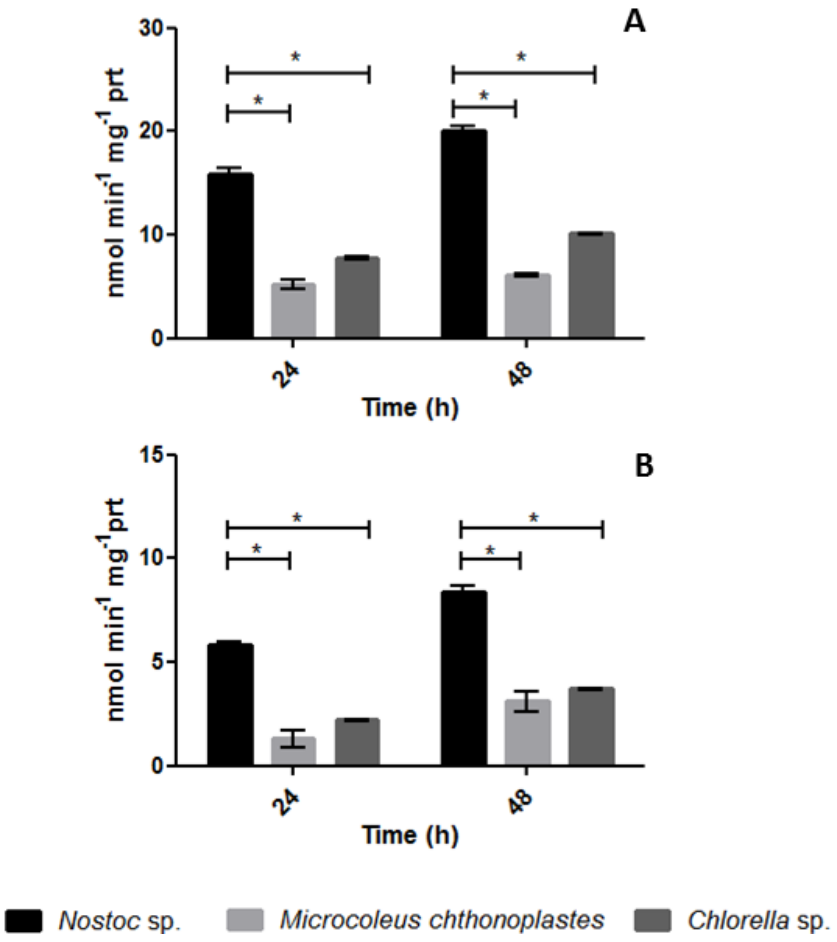

Fig. 7 - (A) GSTs activity and (B) AChE activity in digestive gland of *M. galloprovincialis* fed with *Nostoc* sp., *Microcoleus* sp or *Chlorella* sp. Black columns represent group fed with Nostoc sp,, medium grey columns represent group fed with *Chlorella* sp. and light grey columns represent group fed with *Microcoleus* sp. Bars are the SEM of three replicates (each replicate comprising four individuals of *M. galloprovincialis*). Statistically significant differences were accepted at $p \leq 0.05$. Asterisk bracket indicates significant differences to *Nostoc* sp.

3.3.2.2. Gills

Gills displayed a significantly lower ($p<0.05$) activity in GSTs between mussels fed with cyanobacteria and mussels fed with *Chlorella* sp. (Fig. 8A).

Animals fed with *Nostoc* sp. for 24h and 48h presented a significant increase in

AChE activity ($p<0.05$) in gills when compared to the mussels fed with *Microcoleus* sp or *Chlorella* sp. (Fig. 8B).

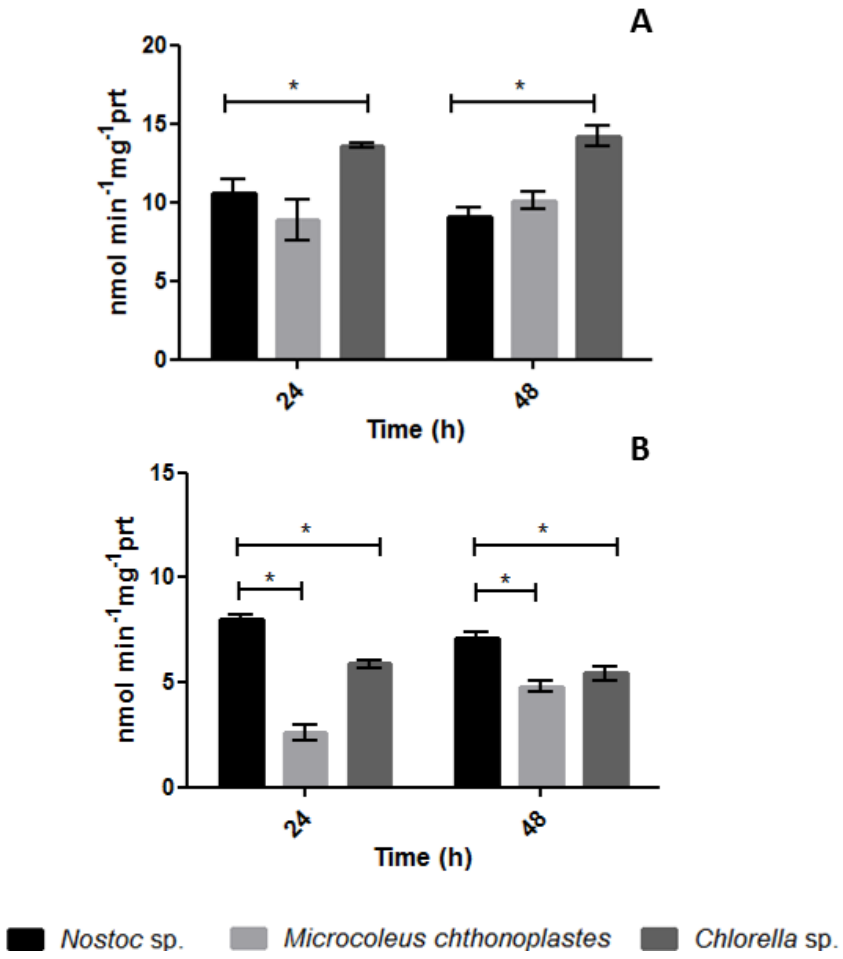

Fig. 8 - (A) GSTs activity and (B) AChE activity in gills of *M. galloprovincialis* fed with *Nostoc* sp., *Microcoleus* sp or *Chlorella* sp. Black columns represent group fed with *Nostoc* sp., medium grey columns represent group fed with *Chlorella* sp. and light grey columns represent group fed with *Microcoleus* sp. Bars are the SEM of three replicates (each replicate comprising four individuals of *M. galloprovincialis*). Statistically significant differences were accepted at $p \leq 0.05$. Asterisk bracket indicates significant differences to *Nostoc* sp.

Moreover, a significant difference in mean activity of AChE and GSTs was observed between the mussels used in both bioaccumulation experiments. While unfed mussels exposed to BMAA standard exhbited activities ranging between 1 and 10 nmol min^{-1} mg^{-1} of protein, mussels fed displayed higher enzimatic activities ranging between 2 and 20 nmol min^{-1} mg^{-1} of protein.

3.3. iGluR expression in *M. galloprovincialis*

3.3.1. Amplification of GLU4 and GLU5

With the designed primers, the GLU4 and GLU5 transcripts were amplified in the gills and digestive gland of the mediterranean mussel, *M. galloprovincialis*. Basal expression of GLU4 and GLU5 genes was identified and quantified in gills from control mussels collected at two different times of the year (Table 6). This preliminary analysis also allowed a comparison between the results obtained with gills collected in Spring and gills collected in Winter and to establish whether the transcripts expression was affected by environmental factors related to seasonal change. According to the results, all genes evaluated showed evident seasonal variations (Table 6).

Table 6 – Mean Ct ± SEM of GLU4, GLU5 and EF-1 α in untreated gills collected from (A) Spring Mussels and (B) Winter mussels.

	GLU4	GLU5	EF-1 A
A	29 ± 1.5	27 ± 0.03	23 ± 1.4
B	32 ± 1.7	32 ± 1.0	27 ± 0.8

3.3.2. Mussels exposed to BMAA standard

In gills, both GLU4 and GLU5 were significantly ($p<0.05$) downregulated after 6h of treatment with BMAA, remaining underexpressed during the complete exposure period (Fig. 9). After depuration, expression values of GLU4 and GLU5 increased significantly ($p<0.05$), and surpassed EF-1 α expression, under the same conditions (Fig. 9).

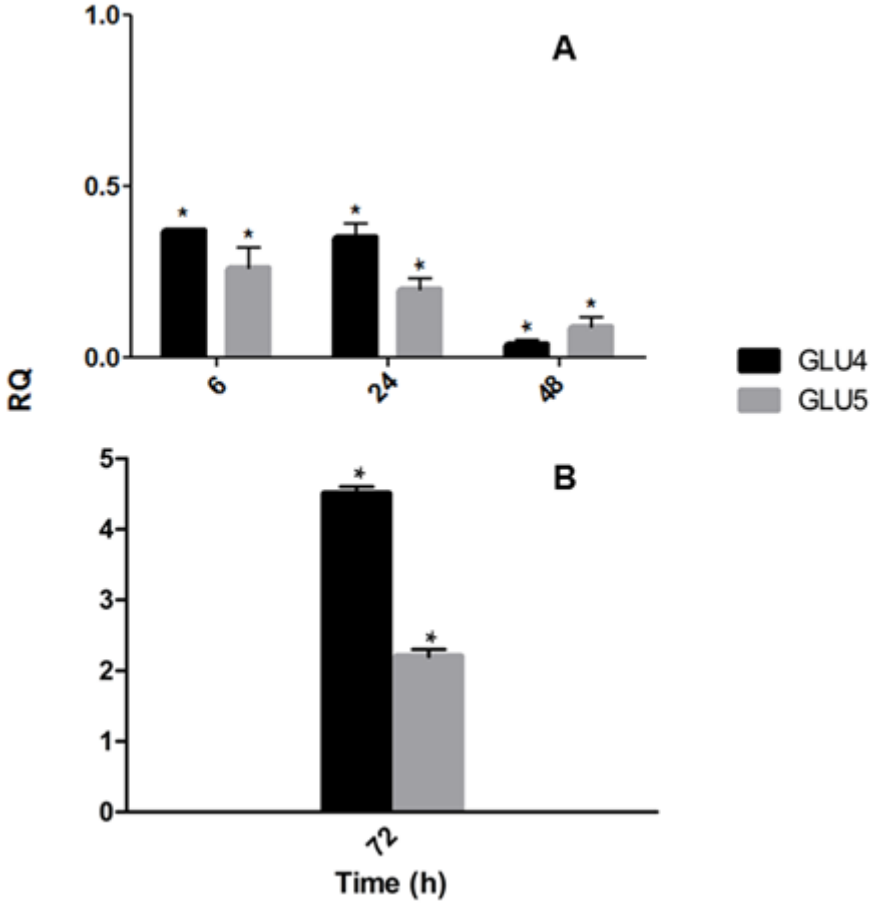

Fig. 9 - Gill changes of GLU4 and GLU5 transcripts expression during (A) exposure to BMAA and (B) depuration period compared with controls. Black columns represent GLU4 transcript and grey columns represent GLU5 transcript (Results are expressed as mean ± SEM. Statistically significant differences were accepted at $p \leq 0.05$; * indicates differences to control). EF-1 α as internal reference.

Digestive gland did not allow a relative quantification analysis, as EF-1 α expression varied between exposed and not exposed mussels in this tissue. Thus, it was not considered a reliable internal reference. After treatment with BMAA, EF-1 α expression was highly depressed (Table 7). GLU4 and GLU5 expression was also affected, but to a lesser extent.

Table 7 - Mean Ct ± SEM values of control and treated mussels digestive gland of GLU4, GLU5 and EF-1 α. Different exposure times have been averaged.

	GLU4	GLU5	EF-1 α
Control	31 ± 2.3	33 ± 1.0	21 ± 0.7
BMAA	36 ± 1.7	36 ± 1.7	36 ± 2.5

4. Discussion

In the last two decades, marine biotoxins have received increasing attention from scientific community, from the development of new analytical methods for toxins detection, to get a better understanding on accumulation and trophic transfer of these molecules in marine organisms, to their mechanisms of toxicity and the generation of more accurate risk evaluations and recommendations (Munday, 2011; Gorbi et al., 2012) . In this study an integrated approach with bioaccumulation of BMAA on mussel and a set of associated subcellular responses was used.

Many discrepancies in published results on BMAA concentration have been reported (Banack et al., 2010; Faassen et al., 2012; Glover et al., 2012). The lack of a standard procedure to determine BMAA in different samples (such as cyanobacteria or marine invertebrates) has been considered an issue. In fact, it was suggested by Faassen et al. (2012) that the inconsistensies observed could be due to the analytical method used. LC-MS/MS has been appointed as the more selective and appropriate method for BMAA detection (Banack et al., 2010; Faassen et al., 2012).

Additionally, matrix effects may be complicating factors in BMAA analysis (Glover et al., 2012). BMAA reactivity and potential to interact with other molecules during the analysis may interfere with the accurate quantification (Li et al., 2010; Glover et al., 2012). In the presence of a complex matrix even more chemical interactions are likely to occur and interfere with the analysis (Li et al., 2010; Glover et al., 2012). Low recovery rates have also been reported when attempting to remove BMAA from saline waters (Glover et al. 2012) and so far, no suitable protocol for BMAA recovery from samples with high concentration of salts has been described. Hence, failure to detect BMAA cannot be considered an absence of the compound.

Concentrations reported in different organisms (phytoplankton, plants, animals) (Faassen 2014) allow the assumption that concentrations ranging 10 µg L^{-1} can be considered environmentally relevant, whereas concentrations above this value are less frequent to come by. Hence, the assessment of the effects of BMAA exposure on enzyme activity and glutamate receptors, such as the one in this study, allows the assessement of toxicity in mussels tissues even when detection of BMAA in the mussel tissues is not possible or reliable.

4.1. Effects of BMAA on AChE and GSTs activity

In this study, levels of enzyme activity in *M. galloprovincialis* were in general, significantly affected by the concentration of BMAA standard and the duration of the exposure.

In the digestive gland, GSTs activity increased during exposure to BMAA and was lowered during depuration. The increase of GST activity was obtained during the accumulation period, for all concentrations, suggesting that this organ is very sensitive, even to low concentrations of BMAA. During depuration, the drop in activity can be explained by the removal of the toxicant from the water. This implies a significant role of GSTs in BMAA detoxification in *M. galloprovincialis*. Although cellular detoxifying pathways involved in BMAA degradation are not clearly understood, phase II detoxification, mainly GSTs-formed conjugates, has been shown to be involved in the degradation process of cyanobacterial compounds (Davies et al., 2005). Formation of the cyanotoxin-GSH conjugate via GSTs that enhances the water solubility of the toxin was observed in different aquatic organisms (Pflugmacher et al., 1998, Adamovský et al., 2007).

In gills, GSTs activity increased during depuration and was lowered during exposure to BMAA. This organ is the major site of uptake of waterborne toxicants, and the presence of BMAA, that initially provoked conjugation action from the enzyme, could have interfered with the mechanisms responsible for regulation of GSTs gene expression, as described for other xenobiotics (Chen and Ramos, 1999; Contardo-Jara et al., 2008). In fact, BMAA, could act in a similar way as MC and okadaic acid, which are known to inhibit protein phosphatases (PP). These PP are involved in cell signaling processes (Rivas et al., 2000), and an inhibition could cause major effects on signal transduction pathways, and consequentially, a disturbance of essential cellular functions (Svensson et al., 2003). During depuration, the GSTs activity increase could be explained by the fact that a rise of the toxin levels could have occurred due to the release of previously bound toxins, from the renewal of PP. This response has been observed for MC (Fernandes et al., 2009). The decrease in activity registered during exposure points to a high vulnerability of the gills to the toxin. Šetlíková and Wiegand (2009) showed that gills of two different fish species had lower GSTs activities and consequently a lower ability to biotransform MC than the respective livers.

AChE activity is commonly used as a bioindicator and there are a number of studies in the literature concerning the effects of environmental organic pollutants, heavy metals and other chemical toxicants on AChE activity in animals (Rao et al., 2005, Richetti et al., 2011; Pereira et al.,2012).

In gills, no apparent effect of BMAA exposure was observed in AChE activity after 48 hours. However, in the digestive gland the results show that, in the tested conditions, the decreased activity of AChE after 48h of exposure to BMAA was reversible after transfer to toxin-free media. However, prolonged exposure to this toxin may impair actions such as feeding activity of the mussels and therefore compromise bioenergetics and general physiological condition (Kankaanpää et al., 2007).

In *M.galloprovincialis* fed with cyanobacterial cells, tissue-specific changes in the enzymatic activity of GSTs and AChE were also observed.

In the digestive gland, the increase of GSTs activity was relevant at 24h and 48h of feeding with *Nostoc* sp. This increase in activity points to a potential stimulation of the detoxification process of cyanobacterial secondary metabolites. Similar results were obtained for liver GSTs activity in several fish species during *Microcystis* blooms in Lake Taihu in China (Qiu et al., 2007). In gills, in contrast to the results observed in the digestive gland, a decrease in activity was registered in response to feeding with *Nostoc* sp. As discussed above, exposure to BMAA, either directly filtered from the water or supplied from toxic cyanobacteria, may inhibit GSTs in gill tissues. However, no differences were observed between mussels fed with *Nostoc* sp. or other strains, that are not producing BMAA, which suggests this effect is not exclusive to BMAA exposure.

M. galloprovincialis is known to present multiple GSTs isoforms divided in three superfamilies: cytosolic, membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) and mytochondrial (Fitzpatrick and Sheehan, 1993). Present data refers to total cytosolic GSTs activity, using CDNB as substrate. Thus, it is not possible to exclude pattern variations from specific isoforms (Regoli and Principato, 1995). Different GSTs isoforms expression seems to be differentially regulated, as different inducers can be related to different isoforms (Hoarau et al., 2006).

The increase in AChE activity was verified in both tissues of mussels fed with BMAA producing cyanobacteria. An increase in AChE activity has been demonstrated as a consequence of exposure to neurotoxic compounds such as aluminum (Kaizer et al., 2010) and ethanol (Rico et al., 2007) as well as cyanotoxin MC (Kist et al., 2012). Recently AChE has been connected to apoptosis as an important regulator (Zhang et al., 2002). In fact, an over-expression of AChE is able to inhibit cell proliferation (Pérez-Aguilar et al., 2015) and promote apoptosis (Zhang and Greenberg, 2012). As it is known, apoptosis often underlies the neurotoxic effects of various compounds such as β-amyloid and prion protein fragments (Forloni et al., 1996). However, the same effect was not observed in mussels exposed to BMAA standard. This study cannot account for the opposite trends verified between mussels exposed to BMAA standard or BMAA

producing cyanobacteria, which compromises the uses of this enzyme as a reliable biomarker of BMAA exposure. It is likely that the rapid and extensive accumulation of secondary compounds in the tissues of the mussels exposed to *Nostoc* sp., caused multiple cellular and physiological responses, including changes in AChE activity. However, the actual mechanism behind the observed fluctuations in AChE activity in *M. galloprovincialis* fed with *Nostoc* sp. is not known. Further studies on potential activity modulating factors are recommended.

Nonetheless, analysis of AChE and GSTs activity as putative markers of exposure to BMAA revealed differences in these parameters in gills and digestive gland from *M. galloprovincialis* mussels exposed to BMAA. In fact, GSTs together with other parameters could be indicators of BMAA exposure. AChE lack of response at lower concentrations in digestive gland, and absence of response in gills in mussels exposed to BMAA, excludes the use of this enzyme as a biomarker.

4.2. Effects of Exposure to BMAA on iGluR Expression

Recent studies in *Mytilus spp.* correlated changes in gene transcription with responses to a variety of environmental stressors: mixtures of crude oil and mercury (Dondero et al., 2006a), copper (Dondero et al., 2006b), benzo(a)pyrene (Brown et al.,2006), okadaic acid (Manfrin et al., 2010) and hypoxia (Hines et al., 2007). Similarly, in this study we identified differentially expressed transcripts in response to BMAA exposure in *M. galloprovincialis* through qPCR analysis.

Given the excitotoxic potential of BMAA it was thought that iGluR gene expression could potentially be used as a biomarker of exposure to BMAA. It was hypothesized that exposure to the toxin would increase the activity of iGluR with consequent increase in their transcription in a concentration-dependent manner. However, the opposite was observed. In gills, the results obtained supported the hypothesis of feedback loop that regulates iGluR expression. When exposed to BMAA standard, a iGluR agonist, it was downregulated. In accordance, when the toxin was removed, this process was reversed. This would mean that despite the lack N-terminal domain, channel activity was not compromised. Nonetheless, full molecular consequences are not yet understood. Thus, discrimination of pharmacologic properties of GLU4 and GLU5 is required to better understand their role *as M. galloprovincialis* glutamate receptors.

Long-term synaptic activity (or its inhibition) has been proved to have an effect on iGluR accumulation at excitatory synapses supporting the idea that feedback

mechanisms are responsible for adjusting neuronal output (Bear, 1995; Miller, 1996). In fact, other studies show that both acute and chronic increases in synaptic activity result in a reduction in postsynaptic iGluR clusters (O'Brien et al., 1998; Lee et al, 2002).

In the environment, the seasonal cycle is a strong determinate of invertebrate physiology (Petrovic et al., 2004; Farcy et al., 2009). Changes in environmental factors resulting from seasonal changes may, therefore, powerfully affect the normal metabolic activities of mussels (Place et al., 2008; Gracey et al., 2008). Therefore, it was expected to find seasonal variation between gene expression of spring mussels and winter mussels, as was observed.

In the digestive gland, it was not possible to estimate the relative expression of GLU4 and GLU5. The development of gonads and gametes that occurs during winter requires a lot of energy, and thus genes non-related to reproduction may be less expressed, during this period (Banni et al., 2011). The reproductive period is an especially energy draining process that induces major changes in the digestive gland (Wilhelm-Filho et al., 2001) In fact, studies have described differences in metabolic activity and genetic expression in this organ , during the reproductive stage of mussels (Seed and Suchanek, 1992; Banni et al., 2011). However, after treatment with BMAA, severe changes in Ct level were observed. The increased Ct levels point to a similar response of downregulation of GLU4 and GLU5, as in gills. However differences in expression EF-1 α cannot be accounted for.

Degeneration of digestive gland tissues and several other histopathological changes could have been induced by exposure to the cyanotoxin, as verified by other studies (Klobuĉar et al., 1997; Auriemma and Battistella, 2004). Moreover, the damaging effects of cyanotoxins have been shown to be enhanced when exposed mussels are spawning (Galimany et al., 2008). Thus, a new experiment with mussels outside of spawning season is necessary to further validate the results obtained. Also, it would be interesting to test the effects of BMAA producing cyanobacteria to confirm the relevance of the results in the environment. Nonetheless, preliminary analysis of iGluR expression in gills of mussels exposed to BMAA standard, as putative biomarkers of BMAA, suggested these genes could be useful markers of BMAA exposure.

5. Conclusions

This study provided a fundamental step in uncovering the molecular responses to BMAA exposure in *M. galloprovincialis*, an organism important in the aquatic ecosystem and present in the diet of many organisms, including humans.

This study showed that an exposure to sublethal concentrations of BMAA in a short period of time had consequences at a molecular and biochemical level, elucidating putative target and/or detoxification mechanisms.

The transcription of iGluR genes can potentially be used as tools to assess BMAA induced toxicity in biomonitoring studies using *M. galloprovincialis.* Nevertheless, further work is needed to better understand the regulatory mechanisms of iGLuR genes as well as their pharmacologic properties and functional role in mussels. Enzymes GSTs were considered a potentially useful marker of BMAA exposure when it is known that *M. galloprovincialis* was exposed to BMAA but the levels are too low for an unequivocal quantification. The lower capacity of response from AChE suggests that this enzyme is a poor marker, though.

Further research will be needed to characterize both natural variability and seasonal changes of *M. galloprovincialis* in responses to BMAA. It is also important to account for possible sinergies of multiple stressors, that may occur in the environment, and affect biomarker modulation. Furthermore, other antioxidant enzymes could also be tested for changes regarding BMAA exposure, thus providing a more complete enzymatic profile for detecting BMAA toxic effects. On a last note concerning BMAA analysis, more investigation should be done to standardize detection and quantification procedures in order to obtain more reliable results.

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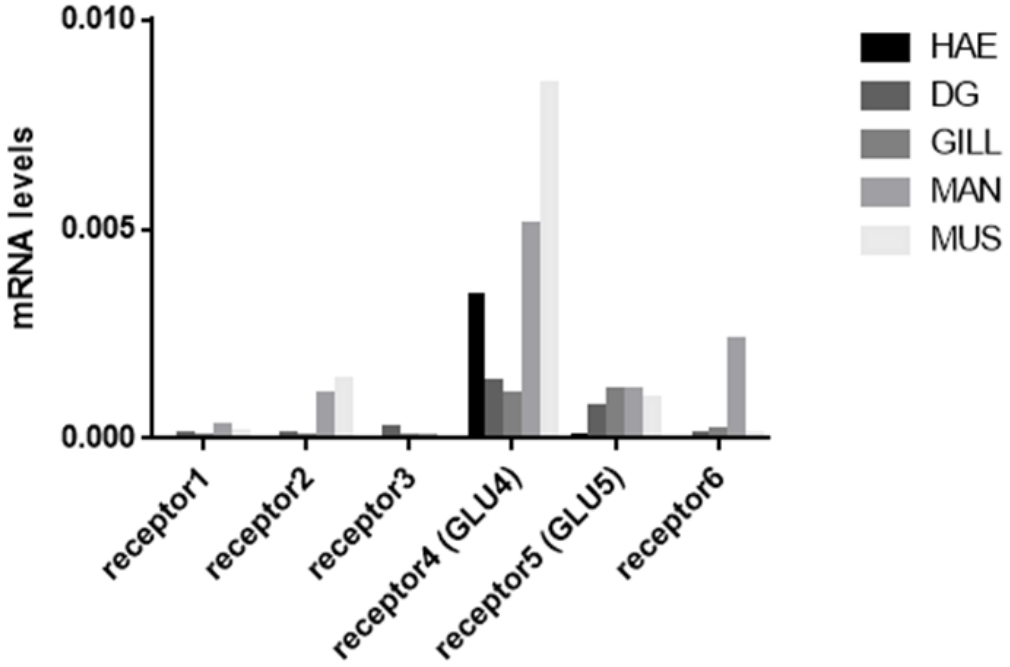
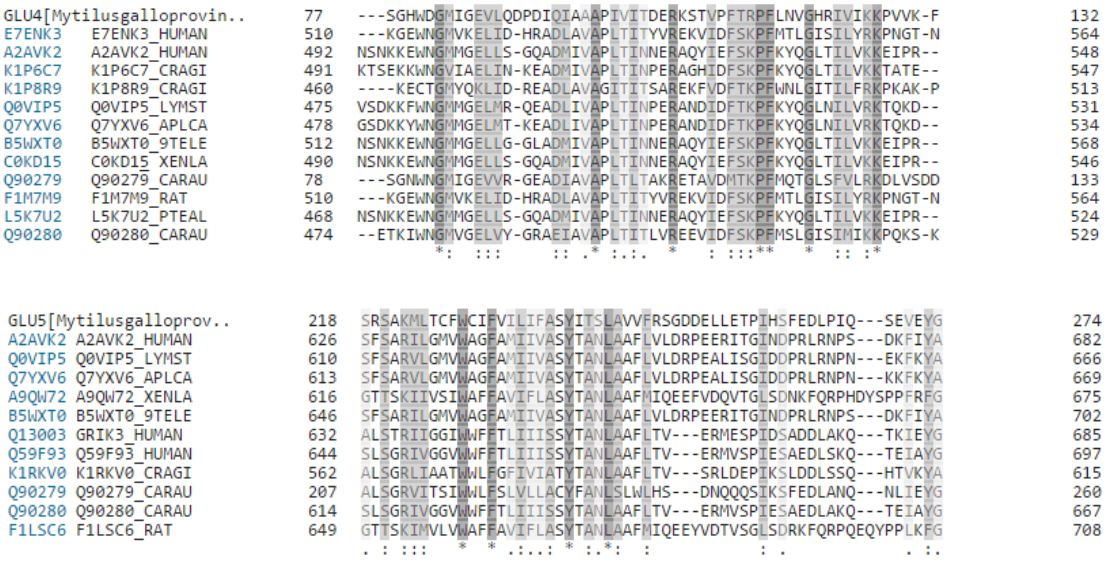
7. Annexes

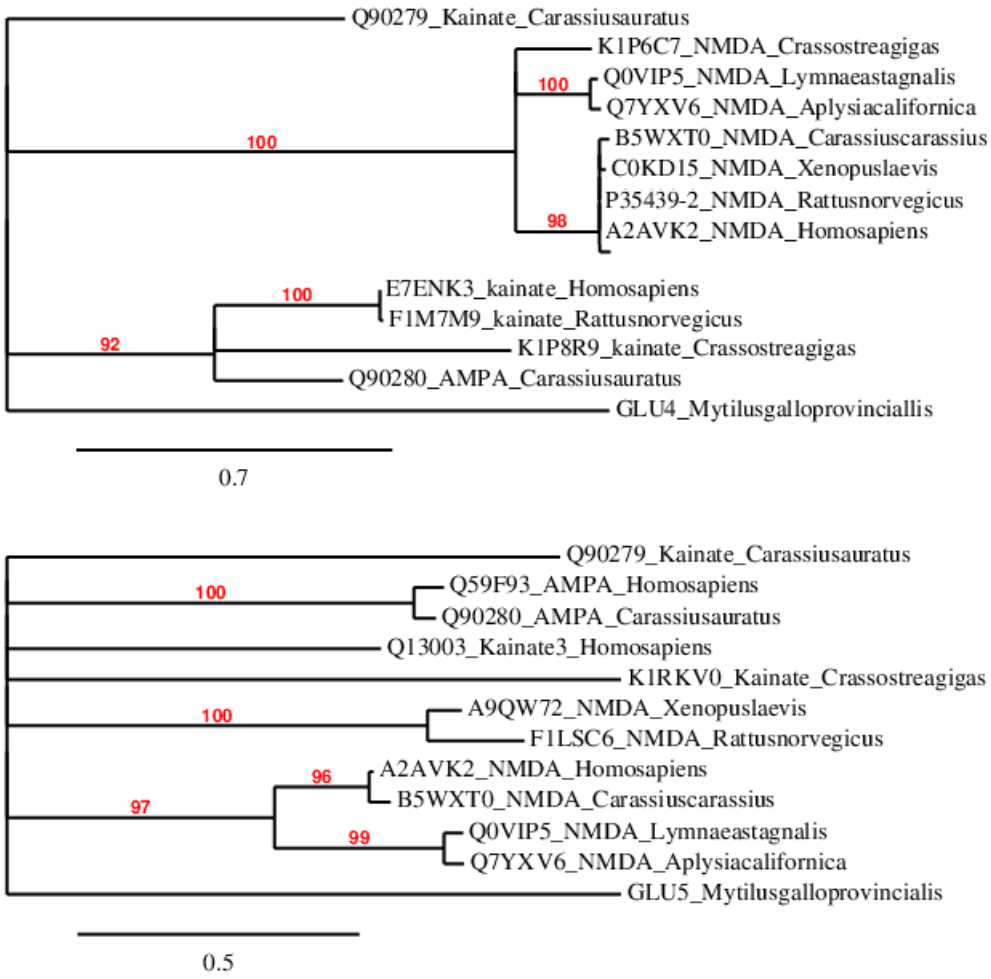


Annex 1 – Expression levels of glutamate receptor sequences (1-6) lifted from Mytibase in *M. galloprovincialis* tissues: HAE – hemolymph, DG – digestive gland, GILL – gills, MAN – mantle, MUS – muscle.
Data shown was previously normalized with EF-1 α expression = 1.

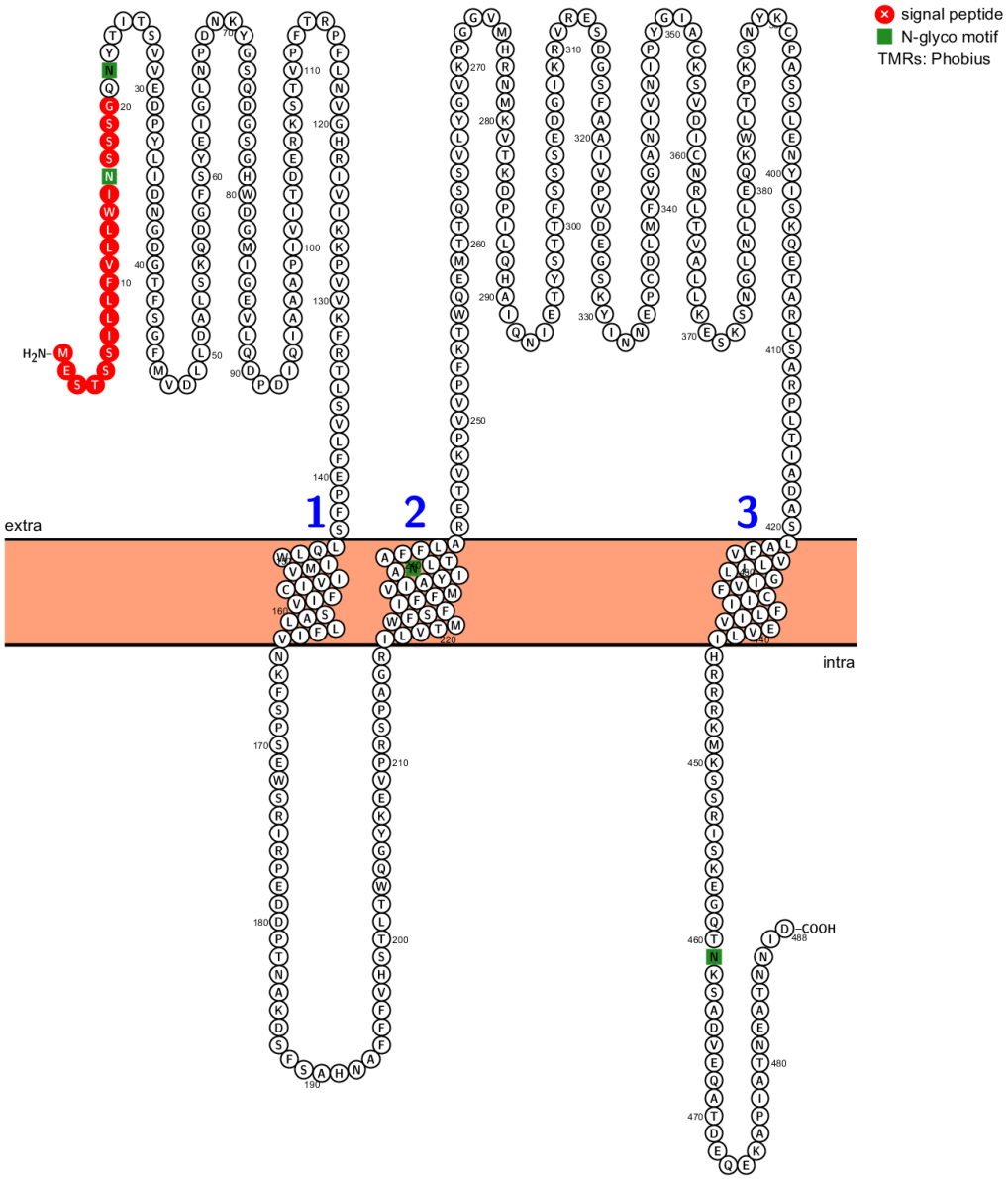
Annex 2 – Domain organization of GLU4 and GLU5 proteins. The proteins start with the glutamate binding domain (Lig_chan/Glu-bd) followed by a Periplasmic Binding Protein (PBPe) domain. Lig_chan, sometimes called the S1 domain, is the luminal domain just upstream of the first, M1, transmembrane region of transmembrane ion-channel proteins, and binds L-glutamate and glycine in the ionotropic glutamate receptor.



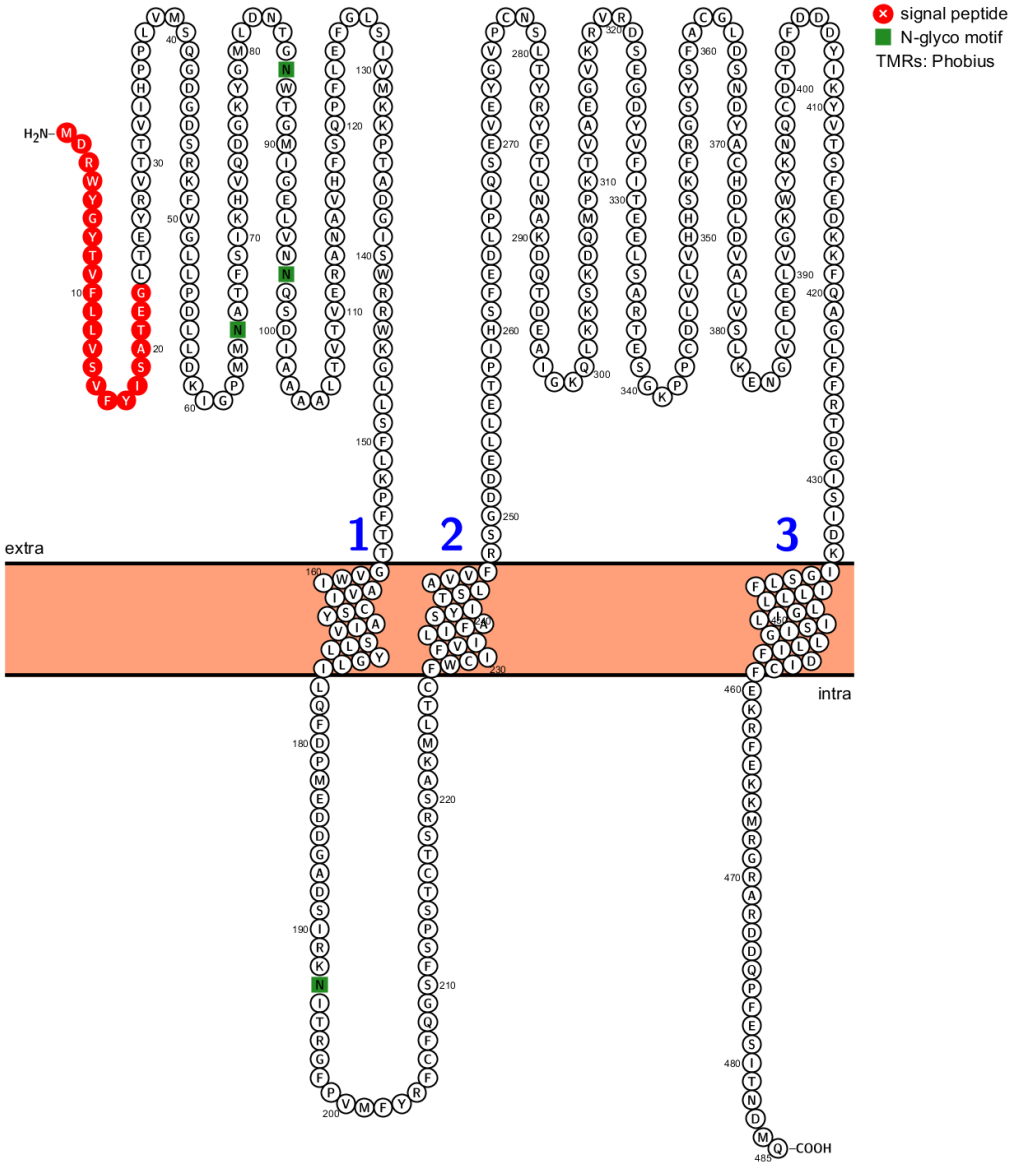
Annex 3 – Sequence alignment produced by Clustal Omega of iGluR from *Homo sapiens, Rattus norvegicus, Xenopus laevis, Carassius auratus, Carassius carassius, Aplysia californica, Lymnaea stagnalis, Crassostrea gigas* and *Mytilus galloprovincialis* (GLU4 and GLU5). Residues that are conserved across all sequences are highlighted in grey. Below the protein sequences is a key denoting conserved sequence (*), conservative mutations (:), semi-conservative mutations (.), and non-conservative mutations ( ).



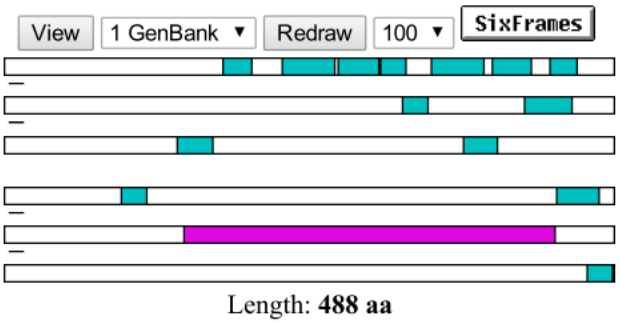
Annex 4 – Phylogenetic Trees of iGluR produced by Phylogeny.fr from *Homo sapiens, Rattus norvegicus, Xenopus laevis, Carassius auratus, Carassius carassius, Aplysia californica, Lymnaea stagnalis, Crassostrea gigas* and *Mytilus galloprovincialis* (GLU4 and GLU5). The GenBank accession number is followed by the name of the host species. Numbers indicate percentage of branch support given by approximate likelihood ratio test (aLRT) and Shimodaira–Hasegawa (SH).



Annex 5 - Plot of transmembranar (TMRs) domains of GLU4 obtained with Protter. TMRs domains previously predicted with Phobius are indicated by numbers 1-3. Signal peptide (red) and glycosylation sites (green) are also shown.



Annex 6 - Plot of transmembranar (TMRs) of GLU5 obtained with Protter. TMRs domains previously predicted with Phobius are indicated by numbers 1-3. Signal peptide (red) and glycosylation sites (green) are also shown.



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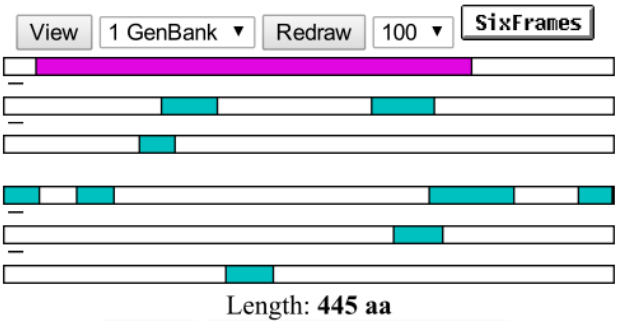
+1	1690	1899	210
+1	1102	1305	204
+2	2054	2242	189
-1	2182	2352	171
+1	1321	1482	162
+1	1930	2082	153
+3	684	827	144
+3	1815	1949	135
+1	865	981	117
+1	2158	2265	108
-1	463	567	105
-3	2303	2404	102
+2	1577	1678	102
+1	1486	1587	102



Frame	from	to	Length
+1	103	1440	1338
-1	1309	1569	261
+2	1133	1324	192
+2	485	661	177
-2	1200	1352	153
-3	683	832	150
+3	417	527	111
-1	229	339	111
-1	1	111	111
-1	1768	1871	105

Annex 7 – Open reading frames (ORF) of GLU4 and GLU5 respectively. Purple bands correspond to the ORF selected to include the primers, posteriorly designed.